ABSTRACT

Structural and Functional Studies on the Fusion and Attachment Envelope Glycoproteins of Nipah Virus and Hendra Virus

Katharine Nina Bossart, Ph.D. 2003

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Nipah virus (NiV) and Hendra (HeV) virus are emerging, biosafety level 4 paramyxoviruses responsible for fatal zoonotic infections of humans from pigs and horses, respectively, and are the prototypic members of a new *Paramyxovirinae* genus called *Henipavirus*. These enveloped, negative-sense RNA viruses infect cells through a pH-independent membrane fusion event mediated through the actions of their attachment (G) and fusion (F) envelope glycoproteins, which are also the principal antigens to which neutralizing antibodies are directed. Understanding the biological and functional features of the viral glycoproteins will help define the characteristics and properties of these novel viruses, and may provide insights into membrane fusion mechanisms, the virus infection process, and towards the development of therapeutics. Here, recombinant vaccinia virus vectors were generated to express the NiV and HeV glycoproteins. Glycoprotein functions and their cellular tropism characteristics were examined with a quantitative assay for membrane fusion. NiV and HeV glycoprotein-mediated fusion could be blocked by virus-specific antisera or synthetic peptides corresponding to the C-terminal α-helical heptad repeats of NiV or HeV F. Both F and G glycoproteins were required for membrane fusion and a broad species and cellular tropism pattern was observed for both HeV and NiV. Further, protease treatment of receptive host cells abolished viral

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Structural and Functional Studies on the Fusion and Attachment Envelope Glycoproteins of Nipah Virus and Hendra Virus

By

Katharine Nina Bossart

Dissertation submitted to the faculty of the Department of Microbiology and Immunology Graduate Program of the Uniformed Services University of Health Sciences F. Edward Hebert School of Medicine In Partial fulfillment of the requirements for the degree of Doctor of Philosophy 2003

To

My sister Mary,

My parents,

(including John and Martha),

Ed, Jay, and Chris

And all

Of my friends

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Chapter 1

Introduction

Emerging and re-emerging infectious diseases

Emerging and re-emerging infectious diseases present a significant threat to the world today. These infectious pathogens include parasites, bacteria, viruses, fungi, and yeast. These agents are capable of infecting a wide range of hosts either directly or indirectly by use of a vector. Some have the ability to spread among and between species with high potential for epidemics. Characterization of these aspects and the relevant virulence factors of the agents are of utmost importance in our quest to develop effective prevention and treatment strategies.

Novel emerging infectious diseases are ones that have never been seen before in any population prior to 20 years ago. This may be because of a true new appearance or because we were unable to diagnose them previously. Many of these new diseases have become major sources of morbidity and mortality, which is most likely related to a non-immune host population. Re-emerging infectious diseases encompass pre-existing diseases with a rapid increasing incidence, geographic expansion, or both. With increasing drug resistance, the global health community must combat more emerging and re-emerging pathogens, with fewer effective weapons available. While developing countries often bear the greatest burden of these infectious threats, the potential of these agents to spread rapidly and ubiquitously means that they present a significant risk to the health and development of all nations. Understanding what the risk factors for emergence are and how these pathogens interact with humans is key in determining how to address these new threats.

Factors in disease emergence

The increase in the epidemiological prevalence of infectious agents determines the emergence or re-emergence of infectious diseases. The fitness of an infectious agent, the human host, and the environment all contribute to the increased prevalence of infectious agents. The susceptibility of the host to an agent and the concentration of an agent in the environment are important factors in disease transmission. Behaviors that change the relationship between the environment and the human host probably have the most to do with emerging infections today. These conducts include personal behavior, the way we manage food supply, our health care system, and the deterioration in public health infrastructures. In addition, many human activities and behaviors have given rise to changes in the global and local environments. Finally, the increase of microbial adaptation and drug resistance has lead to increased pathogen fitness and an increase of emerging and re-emerging diseases.

Environmental reasons for a rise in emerging and re-emerging disease include changes in global and local conditions that can affect the habitat of pathogens and their potential hosts. Examples include but are not limited to, de- and re- forestation, El Nino and heavy rains, discharge of ballast, algal blooms due to fertilizer runoffs, urban crowding, and increased interaction with zoonotic disease agents. For example, recently, changes in El Nino-Southern Oscillation events have had a detectable influence on Rift Valley fever (1). Rift Valley fever is an example of a re-emerging vector-borne disease caused by the bunyavirus, Rift Valley Fever virus (RVFV). Climactic warming has directly affected the prevalence of RVFV by prolonging survival rates of the vector involved in disease transmission.

Cholera epidemics, caused by *Vibrio cholerae* have been linked to specific seasons and biogeographical zones. In addition, the population dynamics of *V. cholerae* in the environment are strongly controlled by environmental factors, such as water temperature, salinity, which are, in turn, controlled by larger-scale climate variability. The study of *V. cholerae* represents a model system of how climate change affects pathogens (2).

Personal human behavior has severely affected the local environment of pathogens giving rise to emergence and re-emergence of disease. Such behaviors include the increased numbers of sexual contacts and illicit drug use, and the increased amount of travel around the world. The human immunodeficiency virus-1 (HIV) is an example of an emerging virus that has become globally widespread due to the increased numbers of sexual contacts. This increase has lead to HIV epidemics in most developing countries throughout Africa, and the number of infected individuals continues to grow to epidemic proportion in other regions of the world such as Thailand, India and China. Intravenous (i.v.) drug use coupled with sharing needles also contributes to the spread of HIV infections, particularly in urban environments but also in rural environments. Recent data demonstrated that HIV infected i.v. drug users exhibit more side effects and complications with anti-viral therapies, and often fail to comply with drug regiments leading to more circulating virus within this population (3). Hepatitis C (HCV) represents another emerging virus where the majority of new infections today occur in i.v. drug users (4). Both HIV and HCV-infected individuals demonstrate how an individual behavior can favor the persistence and or transmission of a virus within the population and lead to its emergence.

Today, individuals do far more traveling around the world than used to be possible. This increase in travel has given rise to the possibility of geographic expansion of both pathogens and their hosts. Dengue fever is the leading arboviral disease in humans. Dengue has become a major public health problem globally where up to 50 million infections occur annually with 500,000 cases of dengue hemorrhagic fever and 22,000 deaths mainly among children. Prior to 1970, only 9 countries had experienced cases of dengue hemorrhagic fever (DHF); since then the number has increased more than 4-fold and continues to rise (World Health Organization). The spreading mechanisms of outbreaks are related to the vector density of the Aedes populations due to climactic factors and the dramatic increase of travel and exposure of non-immune hosts (5).

Although personal behavior does include eating habits, our societies manage food supplies in ways that affect overall public health. In developed nations it appears to be the mass processing and distribution of food, particularly meat, that gives rise to larger more widespread outbreaks of food-borne illnesses. *Enterohaemorrhagic E. coli* (EHEC) are pathogenic, acid-resistant bacteria that have emerged in cattle and are capable of contaminating beef, nearby farm fruit and vegetables, and water supplies, and ultimately infecting humans (6). The combination of poor sanitary conditions and the mass production of beef at large processing plants has facilitated the spread of contaminated products to large batches of meat (7). Because many different communities receive meat from the same processing plant there have been more widespread outbreaks of EHEC. Salmonella and other food related illnesses have increased for similar reasons, as reviewed in (8).

In developed and developing countries, many animals that are farmed for their food value are responsible for transmission of pathogens to humans. In some cases, farm animals can act as intermediate hosts, allowing an agent to evolve and become infectious for humans, in others, the animal may simply serve as an amplifying host. For example, avian influenza viruses primarily infect birds and do not normally propagate in humans. The receptor specificities of avian and human influenza viruses restrict the ability of these viruses to replicate in humans ands birds, respectively. Human practices of farming chickens and ducks in the close proximity of pigs has given rise to new avian-human hybrid viruses that are capable of infecting humans. Recent evidence supports a hypothesis that avian and human influenza viruses are both capable of replicating in the pig (9). Since both of these viruses have an eight-segmented RNA genome, the co-infection of the pig may facilitate reassortment and the creation of new hybrid viruses. In such circumstances the new virus may be able to replicate in humans, but still have avian virus glycoproteins such as the hemaglutinin, which humans lack the immunity to. New pathogenic hybrid viruses normally have an antigenic shift and this mechanism of mutation is different from the antigenic drift of influenza viruses already circulating in the human population.

With the constant existence of new and evolving pathogens, we as hosts are constantly changing, especially with the help of medicine. Importantly, it is necessary to evaluate how disease progression and therapy affects other aspects of host and pathogen dynamics. Currently, with the worldwide epidemic of HIV, and the widespread use of chemotherapy in cancer patients, health systems are coping with large numbers of immunocompromised individuals who are susceptible to infection by many normally

benign microorganisms. Moreover, due to the misuse of antibiotics and adaptation by pathogens, antimicrobial resistance has become a major problem in many hospitals, so infections that were once thought to be under control are now re-emerging as health concerns. Both of these factors directly affect the fitness of the host and the pathogen and represent major concerns when addressing new and re-emerging diseases.

Local environmental circumstances that affect host susceptibilities to disease normally arise from social and economical disruptions or the collapse of public health infrastructures. Moreover, developing countries lack the financial capacity to have research facilities and public health infrastructures and are at a higher risk for the spread of disease. Breakdown of the public health infrastructure due to social disruption was a key element in the reemergence of *Bordetella pertussis* in the former U.S.S.R. during the change of government. Although there was an effective vaccine that prevents whooping cough, there was no funding or infrastructure to administer the vaccine properly to children. Failure to achieve high levels of immunity among children contributed to the epidemic of diphtheria that occurred in the former U.S.S.R. during the 1990s (10).

Another example illustrating a lack in public health support, causing the spread of an emerging infection, is demonstrated by HIV in Africa. The majority of new and current HIV infected individuals live on the continent of Africa and many African nations do not have well-funded public heath programs. In addition, the gross national product (GNP) of most of these countries is a fraction of the developed world. As a consequence, many of the nations that need anti-HIV drugs the most have no means of acquiring them and/or administrating their use broadly. This problem is common in many developing

countries that are facing HIV/AIDS epidemics, with 95% of worldwide HIV infections occurring in these countries (11).

Another new scenario that demands new public health measures is the needed infrastructure locally and globally to deal with an outbreak of disease as a result of bioterrorism. Anthrax, caused by *Bacillus anthracis*, is a zoonotic disease normally associated with sheepherding. As a result of bioterrorism, pulmonary anthrax was responsible for several unusual deaths in the United States in 2001 in Washington, D.C., Florida, New York, and New Jersey. Pulmonary anthrax is a rare disease in the United States, and many physicians have never seen a case. It is of great importance to retrain our doctors to recognize the diseases that could arise from biowarfare agents because the diagnosis of outbreaks of unknown or rare disease is key in containment and treatment regimens.

Emerging zoonotic disease

Any one element does not solely contribute to the emergence or reemergence of a disease, and most cases involve a combination of the factors discussed above. Emergence and re-emergence of zoonotic infections most often have multiple underlying factors. Many new pathogens that are significant causes of morbidity and mortality in humans are zoonotic diseases. A wide variety of animal species, including both domestic and wild, act as reservoirs for these pathogens. The factors that influence the ability of each infectious agent to effectively cross the species barrier and infect new cells and populations are poorly understood. For zoonotic diseases, the increasing proximity of human and animal populations due to the growth of human populations, changes in social

and economical behaviors, including livestock management and food production, and the mobility of animal and human populations, has all contributed to their emergence in humans.

Viruses seem to account for many of the novel emerging zoonotic diseases seen in the world today. Probably the best examples of viral infections that have caused the deaths of many millions of people in the past century and are still emerging and re-emerging are the influenza and AIDS pandemics. These events occurred as a direct result of the introduction of animal viruses into the human population. In nature, influenza viruses are maintained in a variety of hosts including humans, horses, pigs, wild and domestic birds, and sea mammals. Spanish influenza killed more than 20 million people worldwide, and recent genetic studies have suggested the virus was originally derived from birds. Humans have no protective immunity against avian influenza viruses, and thus hybrid human-avian viruses may produce devastating consequences.

HIV-1 and HIV-2 infection and progression to disease impose major burdens on the health and economic status of many developing countries and are increasing in developed nations everyday. Surveys of other animal species revealed that related viruses, the simian immunodeficiency viruses (SIVs), are widespread in a large number of African simian primates where they do not appear to cause disease (12). Phylogenetic analyses indicate that these SIVs are the progenitors of the human viruses, with SIVcpz from the common chimpanzee the progenitor population for HIV-1, and SIVsm from the sooty mangabey monkey the most likely progenitor of HIV-2. Although it is clear that the AIDS epidemic has a zoonotic origin, it is uncertain whether cross-species viral transmission is common among primates or exactly when HIV-1 and HIV-2 first entered

human populations (12). Close contact with monkeys and chimpanzees as humans encroached on their environment likely lead to the transmission of the virus. Today, 42 million people are estimated to be living with HIV/AIDS. Of these, 37.1 million are adults. 19.2 million are women, and 3.2 million are children under 15 (11).

Hemorrhagic fever caused by emerging viral zoonoses have been recognized in three major families: Arenaviridae, Bunyaviridae and Filoviridae. All are negative-stranded RNA viruses, with genomes in two segments, three segments, or non-segmented, respectively (13). Acquisition of these hemorrhagic fevers in humans generally requires close contact with a vertebrate reservoir species, usually rodents (13). Hantaviruses, a species of bunyaviruses, are the causative agents of the zoonotic disease known as hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus pulmonary syndrome (HPS) in the Americas. These pathogens are maintained in the wild by rodent reservoirs and are mainly transmitted to humans via the aerosol route. Human activities such as rodent trapping, farming, cleaning rodent-infested areas, construction work, camping, and hunting, have been implicated in the occurrence of hantavirus disease (14). Significant above average amounts of rainfall in the "four corners" region in the southwest United States coincided with the first outbreak of a hantavirus in the U.S. and was believed to be the main factor contributing to the dense rodent populations and the emergence and re-emergence of the disease (15).

In the case of filoviruses, which includes Ebola and Marburg viruses, the animal reservoir relevant to human infections is currently unknown, but these viruses can infect monkeys, and have contaminated cell cultures prepared from monkeys (13). Although nonhuman primates have been implicated in the introduction of the virus into humans in

several outbreaks, they are not considered likely to represent the reservoir species because of their high mortality with hemorrhagic disease similar to that seen in humans. However, they may serve as a necessary intermediate amplifying host, allowing for selection of a virus that grows to high titers in nonhuman primates, replicating that virus and ultimately transmitting it to humans. This again illustrates how the encroachment of humans into animal environments influences the spread of emerging infectious diseases.

Hendra and Nipah virus outbreaks

Recently, two newly emerging paramyxoviruses have been described that were identified from cases of severe respiratory and encephalitic diseases in animals and humans, known now as Hendra virus (HeV) and Nipah virus (NiV) (reviewed in (16)). The first outbreak of severe respiratory disease in the Brisbane suburb of Hendra, Australia in 1994 resulted in the death of 13 horses and their trainer, and the non-fatal infection of a stable hand and 7 additional horses. At approximately the same time, in an unrelated incident almost 100 km north of Hendra, a 35-year-old man experienced a brief aseptic meningitic illness after caring for and assisting at the necropsies of two horses subsequently shown to have died as a result of HeV infection. Thirteen months later the man suffered severe encephalitis characterized by uncontrolled focal and generalized epileptic-activity and died. A variety of studies that were performed in the evaluation of this fatality, including serology, PCR, electron microscopy (EM) and immunohistochemistry, strongly suggested that HeV was indeed the cause of this patient's encephalitis, and the virus was acquired from the HeV-infected horses 13 months earlier (17). In all, fifteen horses and two people died in the two episodes. At that

time, the source of the emerging virus was undetermined, but more recently, it has been found that approximately 50% of certain Australian fruit bat species, commonly known as flying foxes, have antibodies to HeV and Hendra-like viruses have been isolated from bat uterine fluids and it appears that these animals are the natural host for the virus (18-21). More recently, the nucleic acid sequence of the genes of HeV has been analyzed and compared with those of other paramyxoviruses (22-24). These later studies have confirmed that HeV is a member of the *Paramyxoviridae*, subfamily *Paramyxovirinae*.

Subsequent to these events, an outbreak of severe encephalitis in people with close contact exposure to pigs in Malaysia and Singapore occurred in 1998. The outbreak was first noted in late September 1998 and by mid-June 1999, more than 265 cases of encephalitis, including 105 deaths, had been reported in Malaysia, and 11 cases of encephalitis or respiratory illness with one death had been reported in Singapore (25). This may represent a near 40% fatality rate upon infection. In addition, this outbreak had a tremendous negative economic impact, which continues to date. Although successful, measures taken in the early days of the outbreak resulted in the slaughter of approximately 1.2 million pigs and the virtual closure of the pig farming industry in peninsular Malaysia. EM, serologic, and genetic studies have since indicated that this virus is also a paramyxovirus, and was closely related to HeV. This virus was named Nipah virus (NiV) after the small town in Malaysia from which the first isolate was obtained from the cerebrospinal fluid (CSF) of a fatal human case (26-30). Most patients presented with acute encephalitis, but several individuals had a neurological relapse up to 39 days after an initial mild presentation and one 12-year-old girl relapsed 4 months after virus exposure (31). Thus, with both HeV and NiV, a prolonged period of infection is

possible before serious neurological disease. They also cause systemic infections with an ability to infect the CNS similar to their related morbillivirus cousins. In the case of NiV, the late presentation and IgG subclass response showed similarities to subacute sclerosing panencephalitis (SSPE), a rare late manifestation of Measles virus (MeV) infection (31).

Molecular approaches used in characterizing HeV and NiV played the most important role in distinguishing them as distinctly new paramyxoviruses. Both are unusual among the paramyxoviruses in their ability to infect and cause potentially fatal disease in a number of host species, including humans. Both viruses also have an exceptionally large genome for paramyxoviruses. HeV and NiV are closely related genetically yet distinct from all other paramyxovirus family members. They are more distantly related to viruses in the *Morbilliviruses* genus (32). The reclassification of HeV and NiV into the new *henipavirus* genus was due to their unique biological and genetic features, and they are categorized as biological safety level-4 (BSL-4) pathogens.

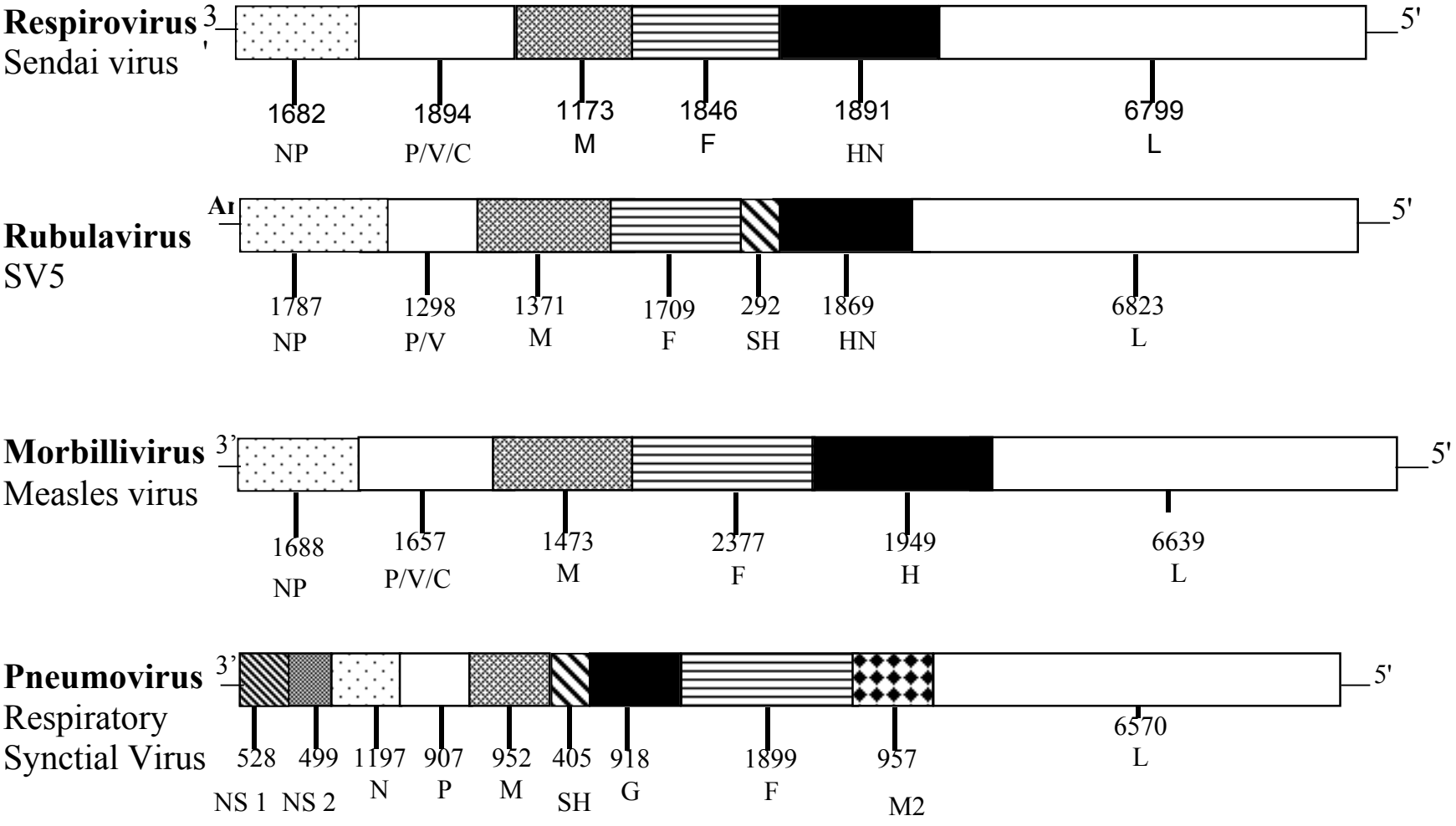
Paramyxoviruses

Paramyxoviruses are negative-stranded RNA, enveloped viruses and encompass a variety of important human and animal pathogens, including measles virus (MeV), mumps virus, Sendai virus (SeV), Newcastle disease virus (NDV), rinderpest virus, canine distemper virus (CDV), human parainfluenza viruses (hPIV) 1-4, respiratory syncytial virus (RSV), and simian virus 5 (SV5) (reviewed in (33)). The genomes of paramyxoviruses, as a group, are generally considered tightly grouped, having sizes in the range of 15.1-15.9 kb. The size and position of the structural genes from the four different genera within the family are depicted in **Figure 1**. The nucleocapsid (NP)

protein serves several functions in virus replication, including encapsidation of the RNA genome into an RNase-resistant nucleocapsid, the template for RNA synthesis, association with the P-RNA polymerase (L) complex during transcription and replication, and, most likely, interaction with the matrix (M) protein during virus assembly. The intracellular concentration of unassembled N is also thought to be a major factor controlling the relative rates of transcription and replication from the genome templates (33). The paramyxovirus matrix (M) protein is the most abundant protein in the virion. In electron micrographs of virions, an electron-dense layer is observed underlying the viral lipid bilayer and this is thought to represent the location of this protein. Fractionation studies of virions indicate that the M protein is peripherally associated with membranes and is not an intrinsic membrane protein (33). The L protein is the least abundant of the structural proteins, about 50 copies per virion. The L gene is the most promoter-distal in the transcriptional map and thus the last to be transcribed. It's low abundance, its large size, and its localization to transcriptionally active viral cores suggested that it might be the viral polymerase. There are five short regions of high homology near the center of these proteins that are also conserved in the RNA-dependent RNA polymerases of other virus families (34, 35).

For the *Paramyxovirinae*, the P gene represents an extraordinary example of a virus compacting as much genetic information as possible into a small gene. The P gene gives rise to a plethora of polypeptides by means of using overlapping reading frames on a single mRNA transcript, by a process of transcription known as "RNA editing", or "pseudotemplated addition of nucleotides". The consequence of the addition of

Figure 1. Genetic Map of typical members of the Paramyxoviridae. An example from each of the four different genera (bold) is shown. NP or N=nucleocapsid; M=matrix; F=Fusion; H or HN or G=attachment, P=phosphoprotein, V=Cysteine-Rich V ORF, C=C protein ORF, SH=small hydrophobic protein, L=RNA-dependent RNA polymerase, NS=non-structural protein.


Respirovirus
Sendai virus
3'
5'
1682
NP
1894
P/V/C
1173
M
1846
F
1891
HN
6799
L
Rubulavirus
SV5
5'
1787
NP
1298
P/V
1371
M
1709
F
292
SH
1869
HN
6823
L
Morbillivirus
Measles virus
3'
5'
1688
NP
1657
P/V/C
1473
M
2377
F
1949
H
6639
L
Pneumovirus
Respiratory
Synctial Virus
3'
5'
528
NS 1
499
NS 2
1197
N
907
P
952
M
405
SH
918
G
1899
F
957
M2
6570
L

pseudotemplated nucleotides to an mRNA is that there is a shift of a translational reading frame into an alternative reading frame, and hence a new protein is translated. Depending on the virus, P, V, and C proteins can be translated. The P protein is a cofactor for the viral RNA-dependent RNA polymerase and is essential for viral RNA synthesis. In Sendai virus (36), hPIV-3 (37), the morbilliviruses (38), and NDV (39), the cysteine-rich V ORF is translated from an mRNA with a single G insertion, whereas in rubulaviruses the V protein is translated from the unedited mRNA. There are seven perfectly conserved cysteine residues in the V ORF, and this domain binds two atoms of Zn^{2+} (40, 41) and is thought to be involved in binding to a host DNA-binding protein (42). Less is known about the various C ORFs, but it appears that they may serve different functions that are genus specific (33).

Paramyxoviruses contain two principal membrane-anchored glycoproteins, which appear as spikes projecting from the envelope membrane of the viral particle when viewed under the electron microscope. One glycoprotein is associated with virion attachment to the host cell, and, depending on the particular virus, has been designated as either the hemagglutinin–neuraminidase protein (HN), the hemagglutinin (H), or the G protein which has neither hemagglutinating nor neuraminidase activities (reviewed in (43)). The other glycoprotein is the fusion protein (F) which is directly involved in facilitating the fusion of the viral and host cell membranes (reviewed in (33)). Following virus attachment to a permissive host cell, fusion at neutral pH between the virion and plasma membranes ensues, resulting in delivery of the nucleocapsid into the cytoplasm. In a related process, cells expressing these viral glycoproteins on their surfaces can fuse

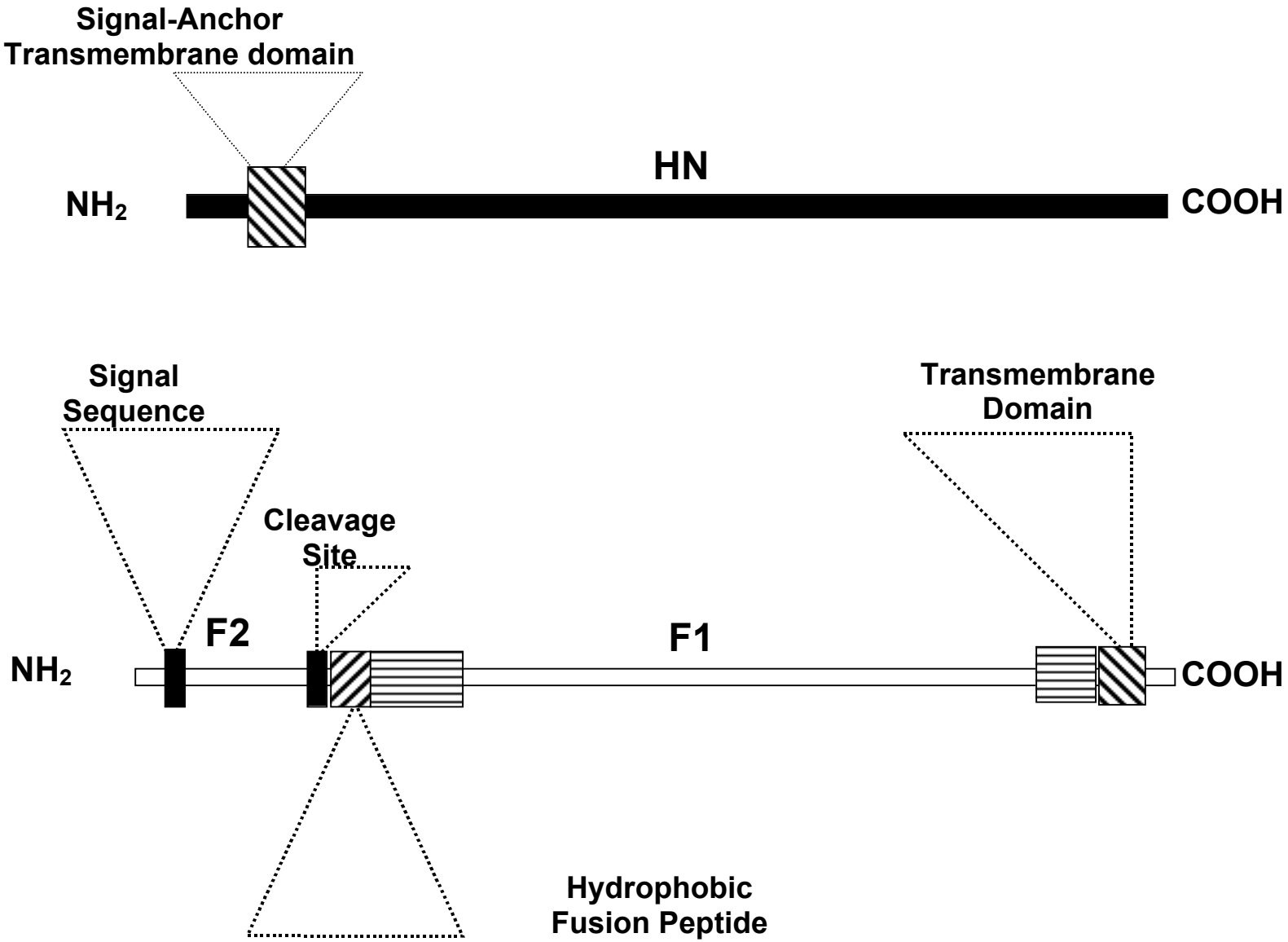
with receptor-bearing cells, resulting in the formation of multinucleated giant cells (syncytia) under physiological or cell culture conditions.

Viruses in the families *Paramyxoviridae*, *Filoviridae*, *Rhabdoviridae*, and *Bornaviridae* are all negative-sense RNA enveloped viruses sharing similar genome organization, replication strategies, and domain structure in the polymerase proteins (reviewed in (32)). These families are grouped in the order Mononegavirales, the first taxon above family level virus taxonomy. The genome size in the Mononegavirales is wide ranging, from 8.9-19.1 kb (44).

Paramyxovirus envelope glycoproteins

The attachment and fusion envelope glycoproteins of paramyxoviruses (**Figure 2**) work together and dictate the entry into cells and, therefore, the tropism of a particular virus. The HN protein is responsible for attachment of the virion to its receptor, sialic acid, on the target cell as is the case for the hPIVs, NDV, SV5 and others. In contrast, the morbilliviruses, like MeV and CDV, have an attachment protein (H) possessing only hemagglutinating activity and do not bind to sialic acid. Earlier work confirmed the predicted interaction between the MeV H glycoprotein and the MeV receptor CD46 using co-immunoprecipitation experiments and soluble CD46 (45). In addition, MeV field isolates as well as vaccine strains have been recently shown capable of utilizing signaling lymphocyte activation molecule (SLAM; CD150) (46). SLAM is also capable of serving as a receptor for several other morbilliviruses, including CDV (47). A third class of paramyxovirus attachment glycoproteins which are possessed by the *Pneumovirinae* such as RSV, are designated G, and have neither hemagglutinating nor neuraminidase

Figure 2. Schematic Diagram of paramyxovirus envelope glycoproteins.


Signal-Anchor
Transmembrane domain
HN
NH2
COOH
Signal
Sequence
Transmembrane
Domain
Cleavage
Site
F2
F1
NH2
COOH
Hydrophobic
Fusion Peptide

activities (reviewed in (43)). The attachment glycoproteins are type II membrane proteins where the molecule's amino (N)-terminus is oriented towards the cytoplasm and the protein's carboxy (C)-terminus is extracellular. The other major envelope glycoprotein is the fusion (F) glycoprotein, and the F glycoprotein of these viruses are more similar, and in all cases they are directly involved in mediating fusion between the virus and host cell at neutral pH. The F glycoprotein of the paramyxoviruses is a type I integral membrane glycoprotein with the protein's N-terminus being extracellular. It shares several conserved features with other viral fusion proteins, including the envelope glycoprotein (Env) of retroviruses like gp120/gp41 of HIV-1, and hemagglutinin (HA) of influenza virus (reviewed in (48)). The biologically active F protein consists of two disulfide linked subunits, F_1 and F_2, that are generated by the proteolytic cleavage of a precursor polypeptide known as F_0 (reviewed in (49, 50)). Likewise, HIV-1 Env and influenza HA are proteolytically activated by a host cell protease, leading to the generation of a membrane distal subunit analogous to F_2 and a membrane-anchored subunit analogous to F_1. In all cases, the membrane-anchored subunit contains a new N-terminus that is hydrophobic and highly conserved across virus families and is referred to as the fusion peptide (reviewed in (51)).

For most paramyxoviruses, efficient membrane fusion requires the presence of both the fusion and attachment glycoproteins, with the exception of detectable F-mediated fusion in the absence of HN seen with the simian virus 5 (SV5) system (52). The details of how the attachment and fusion glycoproteins of the paramyxoviruses function in concert in mediating membrane fusion are not fully understood. For the most part, this interaction is type-specific, where membrane fusion activity mediated by co-

expression (mixing) of the fusion and attachment glycoproteins from different paramyxoviruses (heterotypic) is rarely seen. Although some examples have been noted, the potency of this fusion process in comparison to that mediated by the fusion and attachment glycoproteins from the same virus (homotypic) is considerably less (45, 53). To date, there is more known about the important functional domains of the fusion glycoproteins and their predicted fusogenic conformations that are involved in driving virion-host cell membrane fusion than there is about the attachment glycoproteins. The paramyxovirus fusion proteins are type I membrane glycoprotein trimeric oligomers with considerable hydrophobicity and the attachment glycoproteins are type II proteins with a tetrameric oligomeric configuration (54-57). Both proteins contain several potential N-linked glycosylation recognition sequences. Although it is generally presumed that the attachment protein must contact the fusion protein to induce conformational changes in F, evidence of a physical association between these glycoproteins has been observed with limited success and only with Newcastle disease virus (NDV) (58), human parainfluenza virus (hPIV) (59), and most recently with MeV (60), but these observations have often been with the aid of chemical cross-linking agents. It is hypothesized that following receptor engagement, the attachment protein must somehow signal and induce a conformational change in F that leads to virion/cell fusion (61, 62).

Fusion mechanisms

Paramyxoviruses, like retroviruses, are class I membrane fusion-type viruses. An important feature of the fusion glycoproteins of these viruses is the presence of 2 α-helical domains referred to as heptad repeats that are involved in the formation of a

trimer-of-hairpins structure during or immediately following fusion (63, 64). These domains are also referred to as either the amino (N)-terminal and the carboxyl (C)-terminal heptad repeats, and peptides corresponding to either of these domains can inhibit the activity of the viral fusion glycoprotein when present during the fusion process, first noted with sequences derived from the gp41 subunit of HIV-1 envelope glycoprotein (65, 66). Indeed, HIV-derived fusion-inhibiting peptides have met with clinical success are likely to be the first approved fusion inhibitor therapeutics. Peptide sequences from either the N or C heptads of the F of SV5, MeV, RSV, hPIV, NDV, and SeV have been shown to be potent inhibitors of fusion (67-72). In addition, all known viral envelope glycoproteins are homo- or hetero-oligomers in their mature and functional forms (reviewed in (73)). Multimeric proteins, like these, generally interact over large areas, making structural differences between monomeric subunits and the mature oligomers likely (74). This feature can also translate into differences in antigenic structure and has been shown for a number of proteins, most notably the trimeric influenza HA glycoprotein (75) and HIV-1 gp120/gp41 (76). Indeed, a trimer-specific, potent neutralizing determinant has been mapped to the interface between adjoining subunits of HA, and oligomer-specific anti-HIV-1 Env antibodies have been identified and mapped to conformation-dependent epitopes in gp41 (76). Thus far, all paramyxoviruses, retroviruses, and influenza virus fusion glycoproteins appear to be homotrimers (54, 77-80), and several HN attachment proteins have been shown to be tetrameric, comprised of two homodimers. For example, the NDV HN can form dimers and tetramers on the viral surface (56, 81), and recently the crystal structure of the globular head region of the HN dimer from NDV has been solved (55). Finally, and of importance in understanding

certain aspects of the immune response to these viruses and the development of vaccines, it is the major envelope glycoproteins of these viruses against which virtually all virus-neutralizing antibodies are directed.

Cell Biology of Hendra and Nipah viruses

HeV and NiV both have similar genome organization and a high degree of protein identity (82), and both are classified as paramyxoviruses. Both genomes are 18.2 kb, and they are far closer in size to the Filoviridae than any paramyxovirus including MeV, the largest paramyxovirus. Much of this added length is due to untranslated regions at the 3' end in the six transcription units, again quite similar to Marburg and Ebola filoviruses (32). The N protein of HeV and NiV is 532 amino acids in length with a molecular mass of 58.5 kDa. The N protein has a slightly higher amino acid sequence identity to those of the genus Morbillivirus than to those of other Paramyxovirinae genera, but the level of identity was much lower than that observed within the morbilliviruses (24). The matrix protein is highly conserved between HeV and NiV (82), but significantly different than other paramyxoviruses. Like the N protein, the matrix protein has the most sequence identity with the morbilliviruses (83).

The coding strategy of the P/V/C gene of HeV and NiV is similar to that of Sendai and measles viruses, members of the *Paramyxovirus* and *Morbillivirus* genera, respectively, in the subfamily *Paramyxovirinae*. The P/V/C gene contains four open reading frames, three of which, P, C, and V, have *Paramyxovirinae* counterparts. The P and C proteins are larger and smaller, respectively, than are cognate proteins in members of the subfamily. The V protein is made as a result of a single G insertion during

transcription. The P/V/C genes of HeV and NiV have unique features as compared to other paramyxoviruses. The P proteins are larger, by 100 residues, now the longest known. For HeV, a fourth ORF is encoded between those of the C and V proteins. Although the function of this fourth ORF is unknown, it potentially encodes a small basic protein (SB) similar to those found in some members of the Rhabdoviridae and Filoviridae families.

Although the HeV and NiV envelope glycoproteins are very similar, these proteins are significantly different from other paramyxoviruses (Table 1). The putative ORF for HeV and NiV F and G and their sequence alignments are shown in **Figure 3**. Both the F and G ORFs of HeV and NiV have putative N-linked glycosylation sites (NXS/T). HeV and NiV both possess an F glycoprotein, similar to other paramyxoviruses, that likely mediates membrane fusion. The HeV and NiV F glycoprotein share 83% amino acid identity, and they are identical in the number and location of 6 extracellular potential N-linked glycosylation sites, HeV F has one additional putative site in its cytoplasmic domain (32, 82). The attachment glycoprotein has been designated G, like that of respiratory syncytial virus, because, on the basis of genetic analyses and observations with infectious virus, HeV contains neither hemagglutinating nor neuraminidase activity (84), suggesting that the cellular receptor may not be sialic acid (23). Among the seven residues known to be critical for neuraminidase activity, only one is conserved in the HeV G protein compared with at least six in HN proteins of paramyxoviruses and rubulaviruses and four in H proteins of morbilliviruses. Although the HeV G protein has low sequence homology with

Table 1. HeV and NiV fusion and attachment protein comparison to other paramyxoviruses

	CDV F	MEV F	HEV F	NIV F	
CDV F	***	64.9	29.9	31.7	CDV F
MEV F	33.8	***	30.4	31.1	MEV F
HEV F	67.0	66.7	***	83.0	HEV F
NIV F	65.7	66.9	14.9	***	NIV F

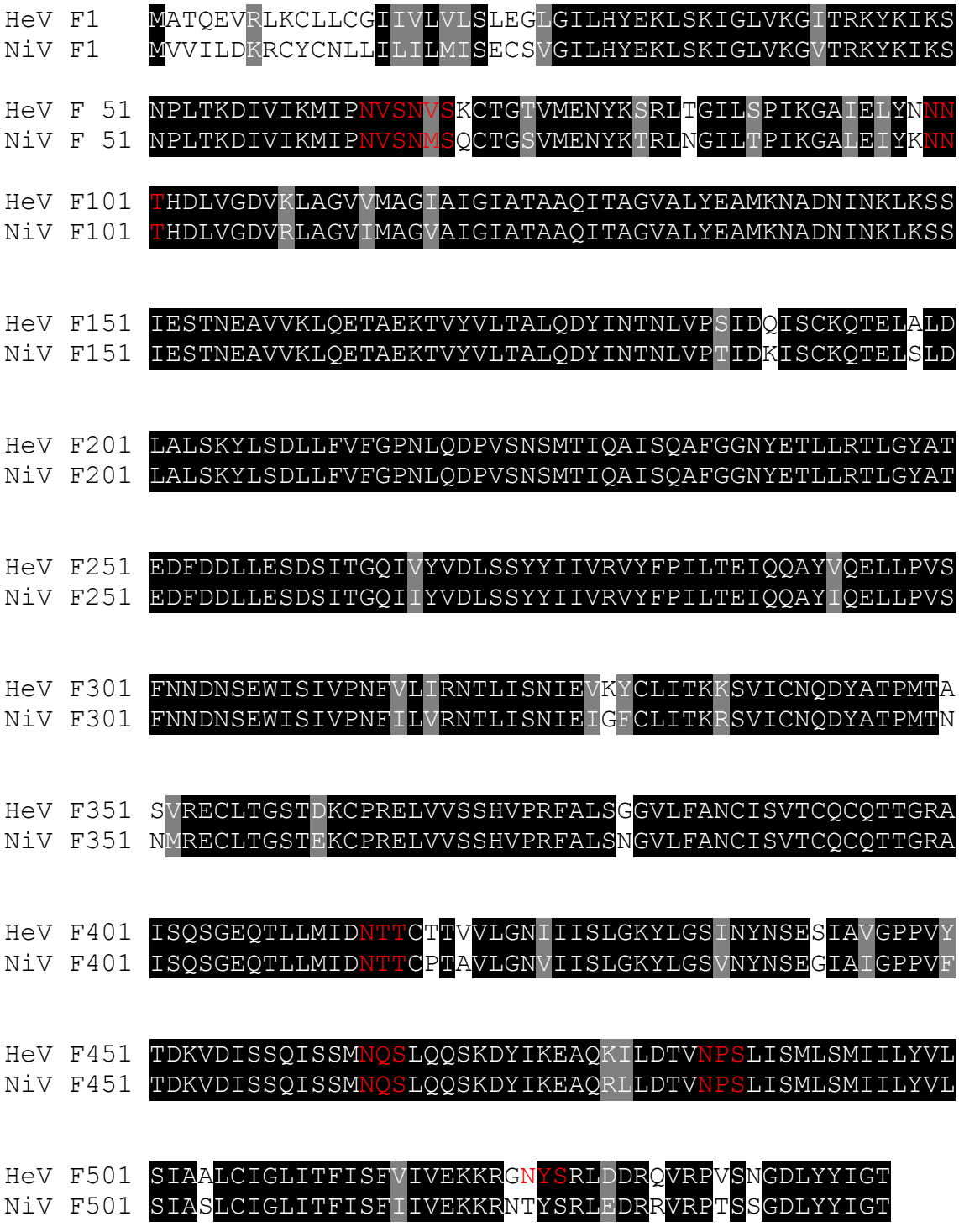
	CDV H	MEV H	HEV G	NIV G	
CDV H	***	19.2	8.1	7.6	CDV H
MEV H	63.1	***	6.7	7.5	MEV H
HEV G	85.3	83.6	***	78.4	HEV G
NIV G	86.4	84.0	21.6	***	NIV G

Percent Similarity in top half of each block
Percent Divergence in lower half of each block

All percent similarities were generated using genebank protein sequences (Accession:

NC_001906 (HeV) and NC_002728 (NiV)) and DNAstar software.

Figure 3. Alignment of the HeV and NiV envelope glycoproteins. The putative protein sequences of F and G were retrieved from genebank (Accession: NC_001906 (HeV) and NC_002728 (NiV)) and analyzed using the Baylor College of Medicine Search Launcher: Multiple Sequence Alignments program. Black background indicates amino acids that are identical, gray background indicate amino acids that are similar, and white background indicates residues that are different amino acids.


HeV F1 MATQEVRLKCLLCGIIVLVLSLEGLGILHYEKLSKIGLVKGITRKYKIKS
NiV F1 MVVILDKRCYCNLLILILMISECSVGILHYEKLSKIGLVKGVTRKYKIKS
HeV F 51 NPLTKDIVIKMIPNVSNVSKCTGTVMENYKSRLTGILSPIKGAIELYNNN
NiV F 51 NPLTKDIVIKMIPNVSNMSQCTGSVMENYKTRLNGILTPIKGALEIYKNN
HeV F101 THDLVGDVKLAGVVMAGIAIGIATAAQITAGVALYEAMKNADNINKLKSS
NiV F101 THDLVGDVRLAGVIMAGVAIGIATAAQITAGVALYEAMKNADNINKLKSS
HeV F151 IESTNEAVVKLQETAEKTVYVLTALQDYINTNLVPSIDQISCKQTELALD
NiV F151 IESTNEAVVKLQETAEKTVYVLTALQDYINTNLVPTIDKISCKQTELSLD
HeV F201 LALSKYLSDLLFVFGPNLQDPVSNSMTIQAISQAFGGNYETLLRTLGYAT
NiV F201 LALSKYLSDLLFVFGPNLQDPVSNSMTIQAISQAFGGNYETLLRTLGYAT
HeV F251 EDFDDLLESDSITGQIVYVDLSSYYIIVRVYFPILTEIQQAYVQELLPVS
NiV F251 EDFDDLLESDSITGQIIYVDLSSYYIIVRVYFPILTEIQQAYIQELLPVS
HeV F301 FNNDNSEWISIVPNFVLIRNTLISNIEVKYCLITKKSVICNQDYATPMTA
NiV F301 FNNDNSEWISIVPNFILVRNTLISNIEIGFCLITKRSVICNQDYATPMTN
HeV F351 SVRECLTGSTDKCPRELVVSSHVPRFALSGGVLFANCISVTCQCQTTGRA
NiV F351 NMRECLTGSTEKCPRELVVSSHVPRFALSNGVLFANCISVTCQCQTTGRA
HeV F401 ISQSGEQTLLMIDNTTCTTVVLGNIIISLGKYLGSINYNSESIAVGPPVY
NiV F401 ISQSGEQTLLMIDNTTCPTAVLGNVIISLGKYLGSVNYNSEGIAIGPPVF
HeV F451 TDKVDISSQISSMNQSLQQSKDYIKEAQKILDTVNPSLISMLSMIILYVL
NiV F451 TDKVDISSQISSMNQSLQQSKDYIKEAQRLLDTVNPSLISMLSMIILYVL
HeV F501 SIAALCIGLITFISFVIVEKKRGNYSRLDDRQVRPVSNGDLYYIGT
NiV F501 SIASLCIGLITFISFIIVEKKRNTYSRLEDRRVRPTSSGDLYYIGT

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HeV G 1   MMADSKLVSLNNNLSGKIKDQGKVIKNYYGTMDIKKINDGLLDSKILGAF
NiV G 1   MPAENKKVRFENTTSDKGKIPSKVIKSYYGTMDIKKINEGLLDSKILSAF

HeV G51   NTVIALLGSIIIIVMNIMIIQNYTRTTDNQALIKESLQSVQQQIKALTDK
NiV G51   NTVIALLGSIVIIVMNIMIIQNYTRSTDNQAVIKDALQGIQQQIKGLADK

HeV G101  IGTEIGPKVSLIDTSSTITIPANIGLLGSKISQCTSSINENVNDKCKFTL
NiV G101  IGTEIGPKVSLIDTSSTITIPANIGLLGSKISQSTASINENVNEKCKFTL

HeV G151  PPLKIHECNISCPNPLPFREYRPISQGVSDLVGLPNQICLQKTTSTILKP
NiV G151  PPLKIHECNISCPNPLPFREYRPQTEGVSNLVGLPNNICLQKTSNQILKP

HeV G201  RLISYTLPINTREGVCITDPLLAVDNGFFAYSHLEKIGSCTRGIAKQRII
NiV G201  KLISYTLPVVGQSGTCITDPLLAMDEGYFAYSHLERIGSCSRGVSKQRII

HeV G251  GVGEVLDRGDKVPSMFMTNVWTPPNPSTIHHCSSTYHEDFYYTLCAVSHV
NiV G251  GVGEVLDRGDEVPSLFMTNVWTPPNPNTVYHCSAVYNNEFYYVLCAVSTV

HeV G301  GDPILNSTSWTESLSLIRLAVRPKSDSGDYNQKYIAITKVERGKYDKVMP
NiV G301  GDPILNSTYWSGSLMMTRLAVKPKSNGGGYNQHQLALRSIEKGRYDKVMP

HeV G351  YGPSGIKQGDTLYFPAVGFLPRTEFQYNDSNCPIIHCKYSKAENCRLSMG
NiV G351  YGPSGIKQGDTLYFPAVGFLVRTEFKYNDSNCPITKCQYSKPENCRLSMG

HeV G401  VNSKSHYILRSGLLKYNLSLGGDIILQFIEIADNRLTIGSPSKIYNSLGQ
NiV G401  IRPNSHYILRSGLLKYNLSDGENPKVVFIEISDQRLSIGSPSKIYDSLGQ

HeV G451  PVFYQASYSWDTMIKLGDVDTVDPLRVQWRNNSVISRPGQSQCPRFNVCP
NiV G451  PVFYQASFSWDTMIKFGDVLTVNPLVVNWRNNTVISRPGQSQCPRFNTCP

HeV G501  EVCWEGSYNDAFLIDRLNWVSAGVYLNSNQTAENPVFAVFKDNEILYQVP
NiV G501  EICWEGVYNDAFLIDRINWISAGVFLDSNQTAENPVFTVFKDNEILYRAQ

HeV G551  LAEDDTNAQKTITDCFLLENVIWCISLVEIYDTGDSVIRPKLFAVKIPAQ
NiV G551  LASEDTNAQKTITNCFLLKNKIWCISLVEIYDTGDNVIRPKLFAVKIPEQ

HeV G601  CSES
NiV G601  CT--
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paramyxovirinae members, the predicted folding pattern of its extracellular globular head is very similar to that of members of the genus Paramyxovirus, with the location of seven potential disulfide bonds absolutely conserved Therefore, the attachment protein of HeV may have high structural similarity in its extracellular globular head, but limited primary sequence homology compared with viruses in the genus Paramyxovirus. The HeV and NiV G glycoprotein share only 78% amino acid identity, yet they both have 8 extracellular potential N-linked glycosylation sites, 7 are identical in location, and the eighth is only shifted by one amino acid. (32, 82). The post-translational modifications of F and G may be critical in determining the native structure of F and G or, perhaps, play an important role in the physical interaction between F and G, and/or act to stabilize the proposed fusogenic conformation of HeV or NiV F. Finally, NiV and HeV possess several biological features which makes them highly adaptable for their use as biowarfare agents; they can be readily grown in tissue culture or embryonated chicken eggs, produce high titers near $1x10^8$ $TCID_{50}$/ml, (85) (Bryan Eaton, per.comm.), are highly infectious and transmitted via the respiratory tract (18, 86), are amplified and spread in livestock serving as a source for transmission to humans, and recent evidence has also indicated that nosocomial transmissibility of NiV from patients with encephalitis to healthcare workers is possible (87, 88)

Specific aims

Understanding the mechanisms of how viruses like HeV and NiV are capable of such an emergence, or mediate host cell infection or cross species transmission, is an important step towards determining how to address these newly emerging pathogens.

Very few laboratories have the capabilities to study these new BSL-4 viruses, and consequently very little is known about their cell biology and what is known is mainly based on genetic information, not functional studies. The subject of this dissertation is the development and use of functional and biochemical assays to examine the cell biology of the envelope glycoproteins of these two new interesting paramyxoviruses in a biosafety level 2 laboratory. Specifically, I sought to:

Specific Aim #1: Establish a functional assay for Hendra virus and Nipah virus envelope glycoproteins**.**

Specific Aim #2: Establish host cell tropism for both viruses using the functional assay.

Specific Aim #3: Generate polyclonal antibodies to F and G glycoproteins for both viruses.

Specific Aim #4: Determine if the envelope glycoproteins from Hendra and Nipah are functionally compatible, and if these proteins can function with other paramyxoviruses, mainly their closest relative the morbilliviruses.

Specific Aim #5: Examine the physical interaction of the F and G envelope glycoproteins, and determine if these interactions correlate with functional fusion rates.

Chapter 2

MATERIAL AND METHODS

Cells and culture conditions. Cells Lines. The following cell lines were obtained from the American Type Culture Collection (ATCC). HeLa cells (ATCC #CCL 2); BS-C-1 (ATCC #CCL 26); CV-1 (ATCC #CCL 70); HuTK$^-$143B (TK$^-$) (ATCC #CRL 8303); RK-13 (rabbit) (ATCC #CCL 37); *Equus caballus* (horse) (ATCC #CCL-57); *Sus scrofa* (pig) (ATCC #CL-101); *Tadarida brasilliensis* (bat) (ATCC #CCL-88). Mouse L2 cells were provided by Anthony Maurelli, Uniformed Services University, Bethesda, MD. Human peripheral blood lymphocytes (PBL) and macrophages were provided by Tzanko Stantchev, Uniformed Services University, Bethesda, MD. Primary chick embryo fibroblasts (CEF) were provided by Norman Cooper, National Institute of Health, Bethesda, MD. 293T, 3T3, cat embryo, and duck embryo cell lines were provided by Jay A. Levy, University of California, San Francisco, San Francisco, CA. A3.01 and A3.02 cell lines were provided by Paul Kennedy, National Institute of Health, Bethesda, MD. Hut 102, MT2, MT4, and CEM human T cell lines were provided by Chou-Zen Giam, USUHS, Bethesda, MD. The human osteosarcoma cell line (HOS) and PM-1 cell lines were obtained from NIH AIDS Research and Reference Reagent Program, the human glioblastoma cell line U373-MG was provided by Adam P. Geballe, Fred Hutchinson Cancer Research Center, Seattle, WA (89). The human head and neck carcinoma cell line, PCL-13, was provided by Vaccinex, Inc., Rochester, NY. HeLa, mouse L2, 3T3, 293T, HOS, U373-MG, and human macrophage cell monolayers were maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% cosmic calf serum (CCS) (HyClone, Logan, UT), and 2 mM L-

glutamine (DMEM-10). PCL-13 cells were maintained in DMEM-10 containing 1mM HEPES buffer pH 7.3 (Quality Biologicals). BS-C-1, CV-1, TK$^-$, and CEF cell monolayers were maintained in Eagle's minimal essential medium (EMEM) (Quality Biologicals) supplemented with 10% CCS, and 2 mM L-glutamine (EMEM-10). Duck embryo, cat embryo, A3.01 cells, A2.01 cells, PM-1 cells, Hut 102 cells, MT2 cells, MT4 cells, and CEM cells and human PBL were maintained in RPMI 1640 (Quality Biologicals) supplemented with 10% CCS, and 2 mM L-glutamine (RPMI-10). Rabbit and horse cell monolayers were maintained in enriched EMEM (Quality Biologicals) supplemented with 10% CCS, 1 mM sodium pyruvate, and 2 mM L-glutamine. Bat cell monolayers were maintained in enriched EMEM containing 0.85 g/L sodium bicarbonate, 2 mM L-glutamine, and 10% CCS. Pig cell monolayers were maintained in Medium 199 (Quality Biologicals) containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, and 3% CCS. All Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere.

Plasmids and Recombinant vaccinia viruses. For expression of recombinant HeV and NiV F and G glycoproteins, the F and G glycoprotein ORFs were subcloned into the vaccinia virus promoter driven expression vector pMC02 (90). The HeV F ORF was initially PCR amplified from plasmid pCP514 (HeV F gene in pFastBac1) (44, 83) using primers 5'- GTTTAAACGTCGACATGGCTACACAAGAGGTCAGG-3' (KB1) and 5'-GTTTAAACGTCGACGATTGTAGTGTATTTTATGTT -3' (KB3). The HeV G ORF was PCR amplified from plasmid pCP484 (HeV G gene in pFastBac1) (23, 44) using primers 5'- GTTTAAACGTCGACCACCATGATGGCTGATTCCAAATTGGTAAGC-3' (KB7) and 5'-

GTTTAAACGTCGACCAATCAACTCTCTGAACATTGGGCAGGTATC -3' (KB8). The NiV F ORF was initially PCR amplified from randomly primed NiV cDNA using primers 5'-CGCGGATCCTCGACAATGGTAGTTATACTTG-3'(NiV-F5-Bam) and 5'-GGTTGAAGCTTCAATCTGAATACACTATGTCC-3'(NiV-F3-Hind) designed according to published NiV genome sequence (91). After gel purification, the PCR product was digested with *BamHI* and *HindIII* and cloned into the same sites of *E. coli* expression vector pRSET-A (Invitrogen). The NiV G ORF was cloned using a similar strategy: PCR primers 5'-CGCGGATCCTTCAAGAAAATGCCGGCAGAA-3'(NiV-G5-Bam) and 5'-GGTTGAAGCTTATGTACATTGCTCTGGTATC-3'(NiV-G3-Hind) were used for initial amplification from random NiV cDNA and the purified digested product was cloned into pRSET-A. The F and G gene coding regions were then transferred by PCR amplification into the vaccinia vector pMC02 using primers 5'-GTCGACCCACATGGTAGTTATACTTGACAAGAGATGTTAT -3' (NVFS) and 5'-GTCGACAGCCGGATCAAGCTTCAATCTGAATACACTATG -3' (NVFAS) for NiV F and primers 5'-CTCGAGCCACATGCCGGCAGAAAACAAGAAAGTTAGATTCGAAAATACT -3' (NVGS) and 5'-CTCGAGTAGCAGCCGGATCAAGCTTATGTACATTGCTCTGGTATC -3' (NVGAS) for NiV G. All PCR reactions were done using Accupol DNA polymerase (PGS Scientifics Corp., Gaithersburg, MD) with the following settings: 94°C for 5 min initially and then 94°C for 1 minute, 56°C for 2 minutes, 72°C for 3 minutes; 25 cycles. These primers generated PCR products for the HeV F and HeV G ORFs flanked by *Sal 1* sites, with an additional *Pme 1* site flanking each *Sal 1* site, and the 5' end of each gene

possessed the sequence CACC upstream of the initial ATG. These NiV subcloning primers generated a PCR product for the NiV F ORF flanked by *Sal 1* sites and the NiV G ORF flanked by *Xho 1* sites. All PCR products were gel purified (Qiagen, Valencia, CA) and subcloned into the TOPO plasmid vector (Invitrogen Corp., Carlsbad, CA). The TOPO HeV F, HeV G, and NiV F construct were digested with *Sal 1* and the TOPO NiV G construct was digested with *Xho 1;* all fragments were gel purified (Qiagen) and subcloned into the *Sal 1* site of pMCO2. For the HeV G and NiV G-myc tagged envelope constructs, HeV G-myc was amplified from the pMCO2 HeV G construct using the following primers:

5’:GTTTAAACGTCGACCACCATGATGGCTGATTCCAAATTGGTAAGC-3’ (GSP) and

5':GTCGACTCACAGGTCTTCTTCGCTAATCAGTTTCTGTTCACTCTCTGAACATTGGGCAGGTATC-3' (HVGMYC). NiV G-myc was amplified from the pMCO2 NiV G construct using the following primers:

5’:CTCGAGCCACATGCCGGCAGAAAACAAGAAAGTTAGATTC-3’ (NVGS) and

5':CTCGAGTAGCAGCCGGATCAAGCTTACAGGTCTTCTTCGCTAATCAGTTTCTGTTCTGTACATTGCTCTGGTATC-3' (NGMYC). For the HeV F and NiV F-HA-tagged envelope constructs, HeV F-HA was amplified from the pMCO2 HeV F construct using the following primers: 5’:

5':GTCGACCACCATGGCTACACAAGAGGTCAGGCTAAAGTGTTTGCTCTGTGG-3' (HVFS) and

5':GTCGACTCAAGCGTAATCTGGAACATCGTATGGGTATGTTCCAATATAATACAGATCACCATTACTGA-3' (HVF HA). NiV F-HA was amplified from the pMCO2

NiV F construct using the following primers: 5': GTCGACCACCATGGTAGTTATACTTGACAAGAGATGTTATTGTAATCT-3' (NVFS) and 5':GTCGACTCAAGCGTAATCTGGAACATCGTATGGGTATGTCCCAATGTAGTAGAGATCCCCACTGCTTGT-3' (NVF HA). The PCR products gave rise to a myc tag at the C-terminus of either HeV or NiV G: N-term-EQKLISEEDL*-C-term, and HA Tags at the C-terminus of either HeV or NiV F: N-term- MYPYDVPDYA *-C-term. HeV G-myc, HeV F-HA, and NiV F-HA were flanked by *Sal I* sites and NiV G-myc was flanked by *Xho I* sites. All PCR products were gel purified (Qiagen, Valencia, CA) and subcloned into the TOPO plasmid vector (Invitrogen Corp., Carlsbad, CA). The TOPO HeV F-HA, HeV G-myc, and NiV F-HA constructs were digested with *Sal 1* and the TOPO NiV G-myc construct was digested with *Xho 1;* all fragments were gel purified (Qiagen) and subcloned into the *Sal 1* site of pMCO2. All constructs were initially screened by restriction digest and further verified by sequencing. The recombinant viruses were then obtained using standard techniques employing *tk*-selection and GUS staining (92). Briefly, CV-1 cells were transfected with all of the pMCO2 constructs individually using a calcium phosphate transfection kit (Promega, Corp., Madison, WI). These monolayers were then infected with Western Reserve (WR) wild-type strain of vaccinia virus at a multiplicity of infection (MOI) of 0.05 PFU/cell. After 2 days the cell pellets were collected as crude recombinant virus stocks. TK^- cells were infected with the recombinant crude stocks in the presence of 25 μg/ml 5-Bromo-2'-deoxyuridine (BrdU) (Calbiochem, La Jolla, CA). After 2 hours the virus was replaced with an EMEM-10 overlay containing 1% low melting point (LMP) agarose (Life Technologies,

Gaithersburg, MD) and 25 μg/ml BrdU. After 2 days of incubation, an additional EMEM-10 overlay containing 1% LMP agarose, 25 μg/ml BrdU, and 0.2 mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GLUC) (Clontech, Palo Alto, CA) was added. Within 24-48 hours blue plaques were evident, picked and subject to two more rounds of double selection plaque purification. The recombinant vaccinia viruses vKB1 (HeV F), vKB2 (HeV G), vKB6 (NiV G), vKB7 (NiV F), vKB11 (HeV G-myc), vKB12 (NiV G-myc), vKB13 (HeV F-HA), vKB14 (NiV F-HA), were then amplified and purified. Bacteriophage T7 RNA polymerase was produced by infection with vTF7-3 which contains the T7 RNA polymerase gene linked to a vaccinia virus promoter) (93). The Escherichia coli *lacZ* gene linked to the T7 promoter was introduced into cells by infection with vaccinia virus recombinant vCB21R-LacZ, which was described previously (94). For cell fusion assays, we either infected cells with the appropriate vaccinia virus encoding the envelope glycoproteins or we transfected cell monolayers with the pMC02-based plasmid constructs containing these genes followed by infection 2 h later with WR vaccinia virus. Transfection of monolayers was performed with DOTAP (Roche Diagnostics Corp., Indianapolis, IN).

Cell-cell fusion assays. Fusion between HeV and NiV glycoprotein-expressing and target cells was measured by a reporter gene assay in which the cytoplasm of one cell population contained vaccinia virus-encoded T7 RNA polymerase and the cytoplasm of the other contained the *E. coli lacZ* gene linked to the T7 promoter; β-galactosidase (β-Gal) is synthesized only in fused cells (95). Vaccinia virus-encoded proteins were produced by incubating infected cells at 31°C overnight (96). Cell-cell fusion reactions

were conducted with the various cell mixtures in 96-well plates at 37°C. Typically, the ratio of HeV glycoprotein-expressing cells to target cells was 1:1 (2 X 10^5 total cells per well, 0.2-ml total volume). Cytosine arabinoside (40 μg/ml) was added to the fusion reaction mixture to reduce nonspecific β-Gal production (96). For quantitative analyses, Nonidet P-40 was added (0.5% final) at 2.5 h and aliquots of the lysates were assayed for β-Gal at ambient temperature with the substrate chlorophenol red-D-galactopyranoside (CPRG; Roche Diagnostics Corp. All assays were performed in duplicate and fusion results were calculated and expressed as rates of β-Gal activity (change in optical density at 570 nm per minute X 1,000) (95). For inhibition by peptides, serial dilutions of peptides were performed and added to envelope glycoprotein-expressing effector cells immediately prior to the addition of target cell populations. For inhibition by specific antibodies, serial dilutions of the various rabbit sera or commercially available monoclonals were performed and added to HeV and NiV glycoprotein-expressing effector cells just prior to the addition of target cells.

Peptide synthesis. Based on analysis of the hydrophobicity plots of the HeV F and G glycoproteins, the most hydrophilic domains were identified and short peptide sequences within these domains were chosen for synthesis and immunization. The following hydrophilic peptide sequences were chosen: CKGITRKYKIKSNPLTKDIVIK (F2), CKSDSGDYNQKYIATKVERGKKDK (G1). The following hydrophilic peptide sequence was chosen for synthesis and immunization based on analysis of the hydrophobicity plot of the NiV G glycoprotein: CKSNGGGYNQHQLALRSIEKGRYDK (NiV G1, amino acids 324-347). Another 44

amino acid peptide corresponding to the C-terminal α-helical heptad domain of HeV F was also synthesized, HQSIQTKVDISSQISSMNQSLQQSKDYIKEAQKILDTVNPSL (FC1). The sequence of peptide FC1 was based on the published sequence (83), which was later corrected (GeneBank Accession: AF017149) (44), and the first 6 residues of the N-terminus of FC1 are irrelevant, however this change is distant from the leucine zipper region and did not affect the peptides activity. A control 44 amino acid peptide derived from the cytoplasmic tail of the Interleukin-2 (IL-2) receptor gamma chain protein, LERTMPRIPTLKNLEDLVTEYHGNFSAWSGVSKGLAESLQPDYS (IL2Rg) was used as the irrelevant control. The following peptide sequences corresponding to the exact C-terminal α-helical heptad domains of the HeV F and NiV F glycoproteins were chosen for synthesis: PPVYTDKVDISSQISSMNQSLQQSKDYIKEAQKILDTVNPSL (HeV FC2), PPVFTDKVDISSQISSMNQSLQQSKDYIKEAQRLLDTVNPSL (NiV FC1). A scrambled version of the HeV FC2 42 amino acid peptide was also synthesized for use as a control, YVKTLKPDVSISQSMIQLQSKPYQIEQKSNDLTNSPVSDIDA (ScHeV FC2). Synthesis of each peptide was accomplished on an Applied Biosystems Model 433 Peptide Synthesizer using HBTU/HOBt activation on a hydroxmethlphenoxymethyl-copolystyrene-1% divinylbenzene resin (97-99). Upon synthesis completion, the resin was washed twice with dichloromethane followed by 3 washes with methanol and allowed to dry. Cleavage of the peptide from the resin was obtained using Reagent R (90% trifluoroacetic acid, 5%thioanisole, 3% 1,2-ethanedithiol and 2% anisole) at room temperature for 3 hours. The peptide was isolated from the mixture by vacuum filtration through a sintered glass funnel into cold ethyl ether that permitted precipitation. Peptide and ether were transferred to a 50 ml centrifuge tube and

centrifuged. The peptide pellet was resuspended in cold ether and centrifuged three separate times to remove residual scavengers and acid. Following the third wash the pellet was allowed to dry completely. Once dry, the peptide was resuspended in 95% water/5% CH_3CN, the pH adjusted to ~7 using dilute NH_4OH, frozen at –20°C, and lyophilized.

Polyclonal antibodies. Peptide-specific rabbit sera to the HeV F and G and NiV G glycoproteins were prepared with the Imject Maleimide Activated KLH Kit (Pierce, Rockford, IL). The F2, HeVG1, and NiV G1 synthetic peptides were reduced with 250 μM dithiothreitol (DTT) (ICN Biomedicals, Cleveland, OH) for 1 hour at 37°C prior to conjugation. Excess DTT was removed using 1800 MW gel exclusion columns (Pierce). 1.56 mg of recovered HeVG1 was conjugated to 2 mg of activated KLH, 1.25 mg of recovered F2 was conjugated to 2 mg of activated KLH and 1.18 mg of recovered NiV G1 was conjugated to 2 mg of activated KLH. Conjugates were purified using a 5000 MW gel exclusion column (Pierce). Each peptide-KLH conjugate was stored in 200 μg aliquots at -80^{0}C. 200 μg F2-KLH conjugate containing 1X RIBI (RiBi Immunochem Research Inc., Hamilton, MT) in 1 ml PBS, 200 μg HeV G1-KLH conjugate containing 1X RIBI in 1 ml PBS, PBS and 200 μg NiV G1-KLH conjugate containing 1X RIBI in 1 ml PBS were administered independently to three rabbits as follows: 0.05 ml intradermal in six sites, 0.3 ml into each hind leg and 0.1 ml subcutaneous in the neck region. Equivalent boosts were given on days 28, 56, and 84. Test bleeds were collected on day 35, large bleeds were collected on days 63 and 91. In addition, sera from rabbits

immunized with gamma-irradiated HeV (anti-IrHeV) or NiV (anti-IrNiV) were also used in some experiments.

ELISA. High-binding 96-well flat bottom plates (Immunlon 2, Dynex Technology) were coated with 250 ng peptide/well in a 50 μl volume of coating buffer (6.05 ml 1 M $NaHCO_3$ + 2.28 ml 1 M Na_2CO_3) overnight at 4^0C. Plates were washed three times with wash buffer (PBS/0.05% Tween-20), and 150 μl blocking buffer (PBS/4% gelatin/ 0.05% Tween-20) was added per well and incubated at 37^0C for 1 hour. Sera were serially diluted during this time in PBS/1%gelatin/0.03%Trition-X 100. Plates were washed three times with wash buffer, and 100 μl of each serum dilution was added in duplicate and incubated at 37^0C for 1 hour. Plates were washed three times with wash buffer. HRP-conjugated goat anti-rabbit IgG (Accurate Chemical and Scientific company) was diluted 1:10,000 in PBS/1%gelatin/0.01%Trition-X 100 and 100 μl was added to each well and incubated at 37^0C for 1 hour. Plates were washed three times with wash buffer and developed with the ABST substrate kit (Roche Diagnostics). The absorbance of each well was measured at 405 nm using a Dynatech plate reader. For competition ELISA sera were mixed with a 25 molar excess of peptide for 1 hour prior to addition to the plate.

Metabolic labeling and immunoprecipitation. For labeling of HeV and NiV glycoproteins expressed by recombinant vaccinia viruses, HeLa cells were infected at a moi of 10 PFU/cell. At 6 h post-infection, monolayers were washed, overlaid with methionine and cysteine-free minimal essential medium (MEM) (Life Technologies)

containing 2.5% dialyzed fetal calf serum (FCS) (Life Technologies) and 100 μCi of [^{35}S] ProMix/ml (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated overnight. Lysis of cells was performed in 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Triton X-100 and the nuclei removed by centrifugation. Typically, 0.5-1.0 μl of rabbit antisera or normal rabbit serum, or 2 μg of anti-myc (9E10) or anti-HA (12CA5) (Roche Molecular Biochemicals) monoclonal antibodies were utilized per immunoprecipitation. Incubations for at least 1 h at 4 ^{0}C were followed by addition of protein G-Sepharose for at least 30 min at room temperature. Complexes were washed twice with lysis buffer (100 mM Tris-HCl (pH7.5), 100 mM NaCl, 1% Triton X-100) and once with DOC buffer (100 mM Tris-HCl (pH8.0), 100 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and visualized by autoradiography.

Western blot analysis. HeLa cell monolayers were infected overnight at a moi of 10 with vaccinia virus encoding wild-type or epitope tagged, HeV F, HeV G, NiV F or NiV G. Cells were extracted with 1% Triton X-100 in 100 mM Tris-HCl, pH 8.0, 100 mM NaCl and the nuclei removed by centrifugation. Lysates were either examined directly, or following immunoprecipitation as described above. Samples were prepared by boiling in sample buffer containing 2-mercaptoethanol. Extracts were loaded onto a 10% SDS-PAGE gel. Following transfer to nitrocellulose paper, the blot was probed with peptide-specific rabbit antisera or 2 μg of anti-myc or anti-HA monoclonal antibodies. The blot was then incubated with HRP-conjugated goat anti-rabbit IgG (Pierce) or and HRP-

conjugated goat anti-mouse IgG (Pierce) and developed with the SuperSignal chemiluminescence kit (Pierce).

Chapter 3

Functional Expression and Membrane Fusion Tropism of the Envelope Glycoproteins of Hendra Virus

RESULTS

Expression of Hendra virus F and G glycoproteins. HeV is classified as a zoonotic, biosafety level 4 (BSL-4) agent and thus its manipulation under laboratory conditions is highly restricted. To readily examine the biochemical and functional properties of the virus' envelope glycoproteins, the viral proteins responsible for host cell attachment and virion entry, we employed the vaccinia virus-based recombinant expression system (92). For the production of recombinant expressed HeV envelope glycoproteins, the HeV F and G ORFs (23, 44, 83) were subcloned into the vaccinia virus promoter driven expression vector pMC02 (90) and recombinant viruses were prepared using standard techniques as detailed in the Materials and Methods. To develop reagents to detect the HeV envelope glycoproteins biochemically, anti-F and anti-G peptide-specific rabbit antisera were prepared (see Materials and Methods). These antisera were titered using a peptide-specific ELISA and specificity was further analyzed by competition ELISA. Shown in **Figure 4** are the hydrophobicity plots for HeV and NiV F and G. Regions chosen for synthesis and immunization are represented by double-ended arrows. The F_2 peptide sequence chosen synthesis for HeV F was identical in NiV F. **Figure 5** and **Figure 6** illustrate that sera are specific for the antigen used. The HeV envelope glycoproteins F and G were produced in cell culture by either transient transfection with the appropriate plasmid construct or by infection with recombinant vaccinia virus.

Figure 4. Hydrophobicity plots of HeV F and G and NiV G. The putative ORF sequences for the HeV F, HeV G and NiV G were analyzed using Macvector 6.5 software. Janin hydrophobicity plots were generated with the positive values indicating highly hydrophobic regions, and the negative values representing hydrophilic stretches. Hydrophilic stretches are more likely to be surfaced-exposed in the native protein, so these domains were chosen for antigen design. The arrows represent peptide sequences within the above plot that were chosen as antigens. For exact peptide sequence, conjugation and immunization protocols see Materials and Methods section. NiV F had a hydrophobicity plot very similar to HeV F, and in fact, the peptide sequence chosen for HeV F_2 was conserved in NiV F, therefore, an additional antigen was not designed.

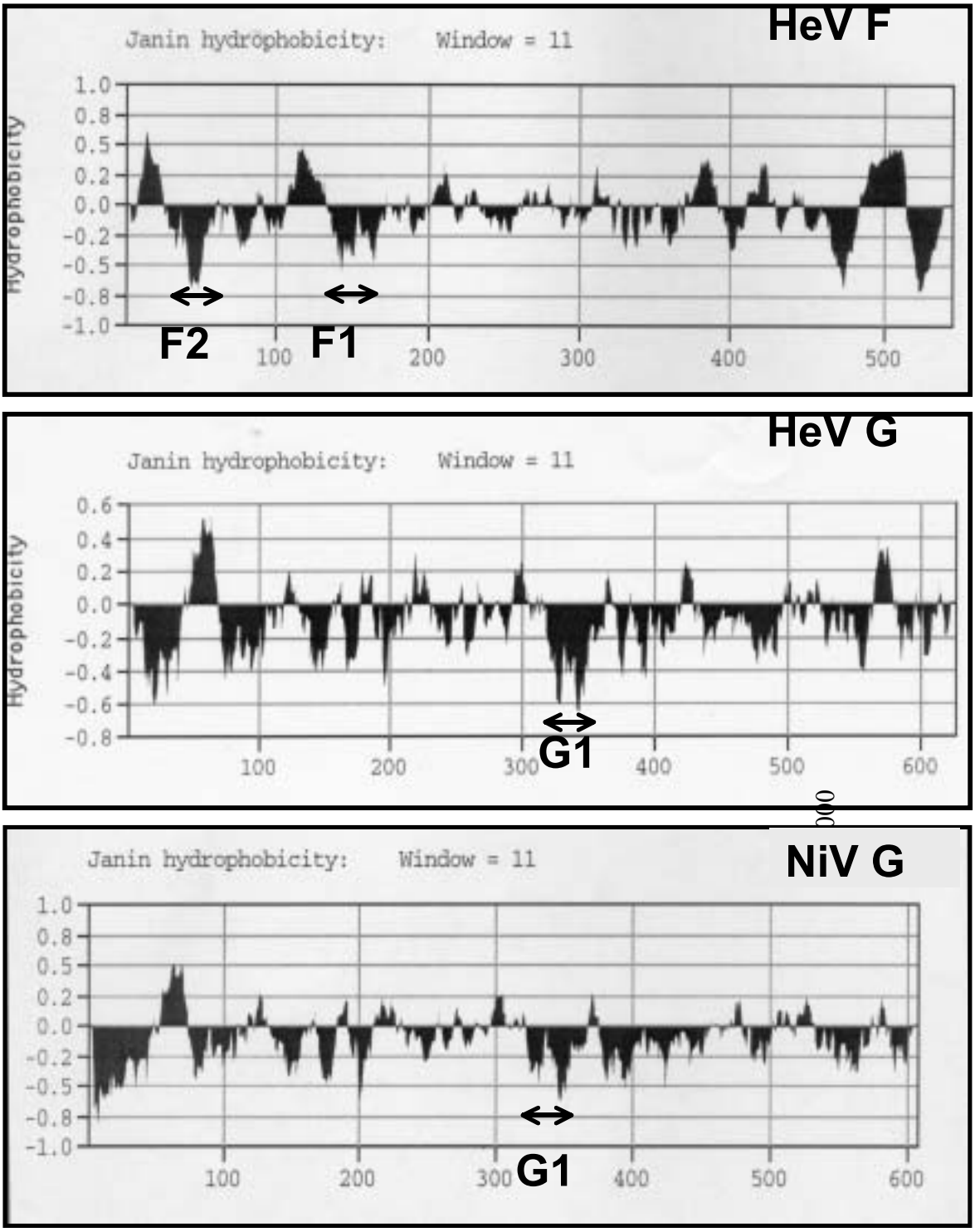
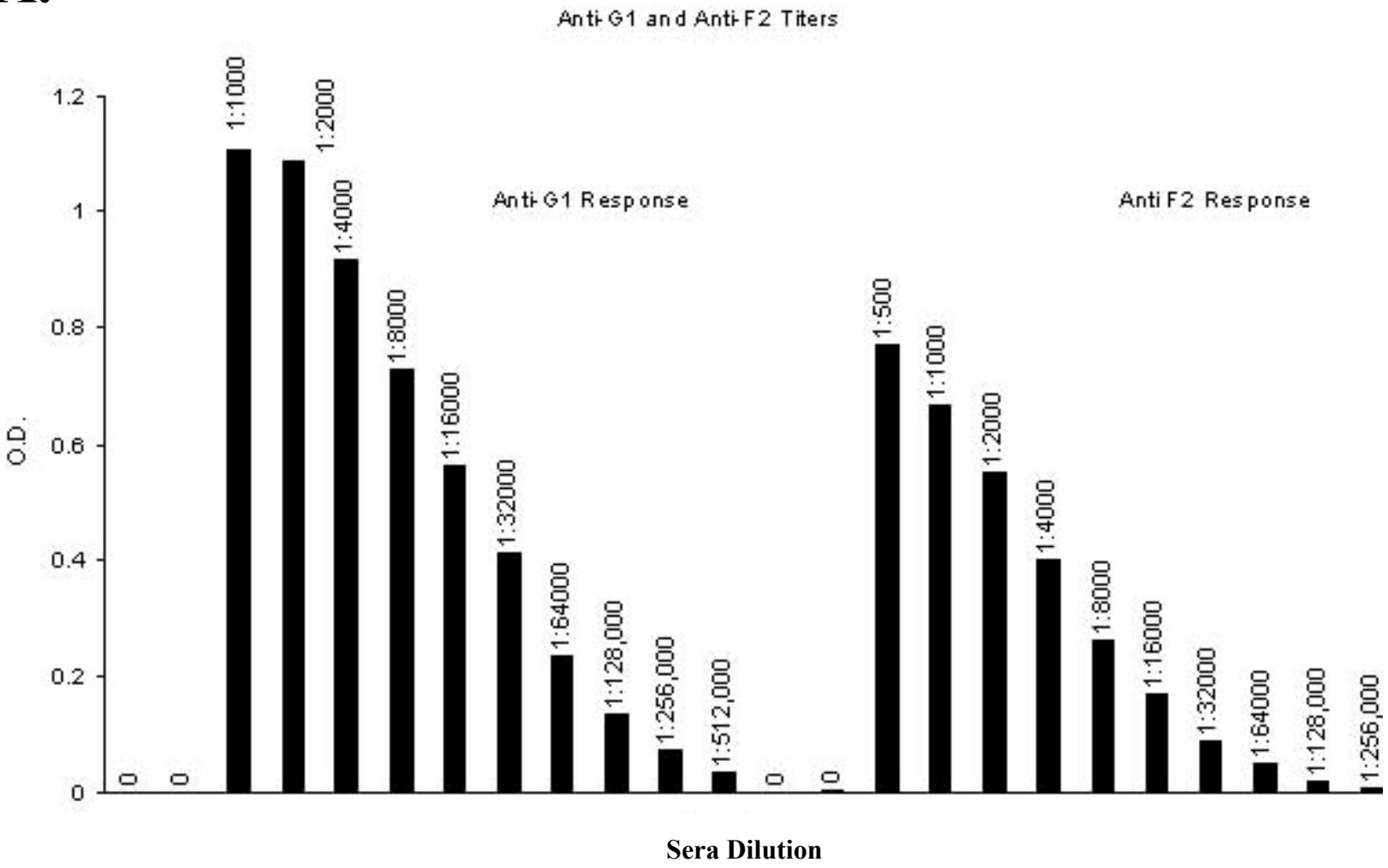
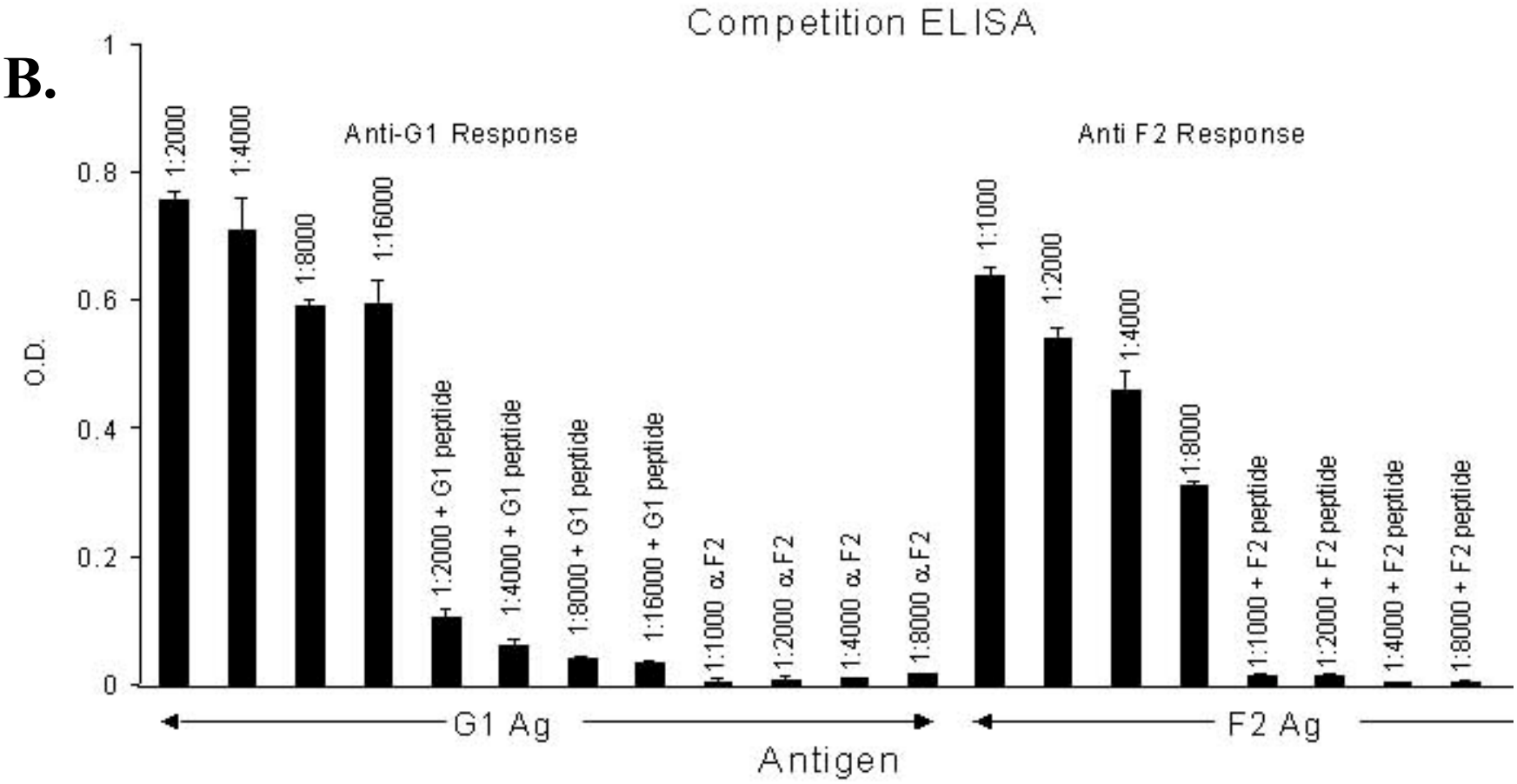

HeV F
Janin hydrophobicity: Window = 11
Hydrophobicity
1.0
0.8
0.5
0.2
0.0
-0.2
-0.5
-0.8
-1.0
F2
F1
100
200
300
400
500
HeV G
Janin hydrophobicity: Window = 11
Hydrophobicity
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
G1
100
200
300
400
500
600
NiV G
Janin hydrophobicity: Window = 11
1.0
0.8
0.5
0.2
0.0
-0.2
-0.5
-0.8
-1.0
G1
100
200
300
400
500
600

Figure 5. ELISA and Competition ELISA Results for HeV specific serum. A. Sera were collected after the second boost and tested against the homologous peptide at different sera dilutions. The two "0" 's represent either no antigen or no antisera and are internal controls for the ELISA assay. B. Competition ELISA. The antisera were diluted and pre-mixed with 100 molar excess of analogous peptide for one hour prior to ELISA. In addition, the anti-F_2 antiserum was tested against the G1 antigen at different dilutions to ensure specificity. Similar analysis was done with the anti-G1 antiserum (data not shown). This experiment was done twice.

A.


Anti-G1 and Anti-F2 Titers
Anti-G1 Response
Anti F2 Response
O.D.
1.2
1
0.8
0.6
0.4
0.2
0
0
0
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
1:128,000
1:256,000
1:512,000
0
0
1:500
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
1:128,000
1:256,000
Sera Dilution

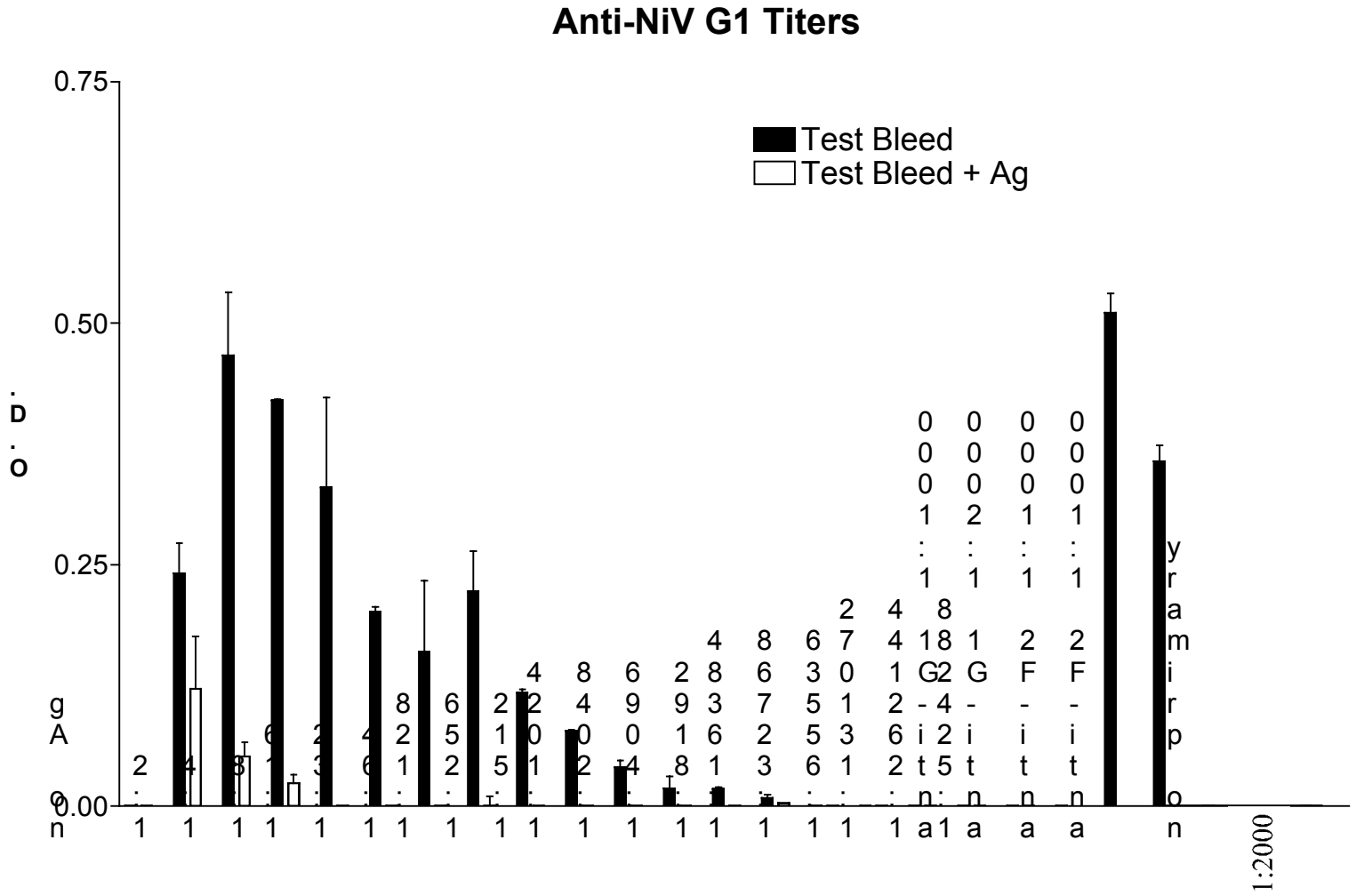
B.



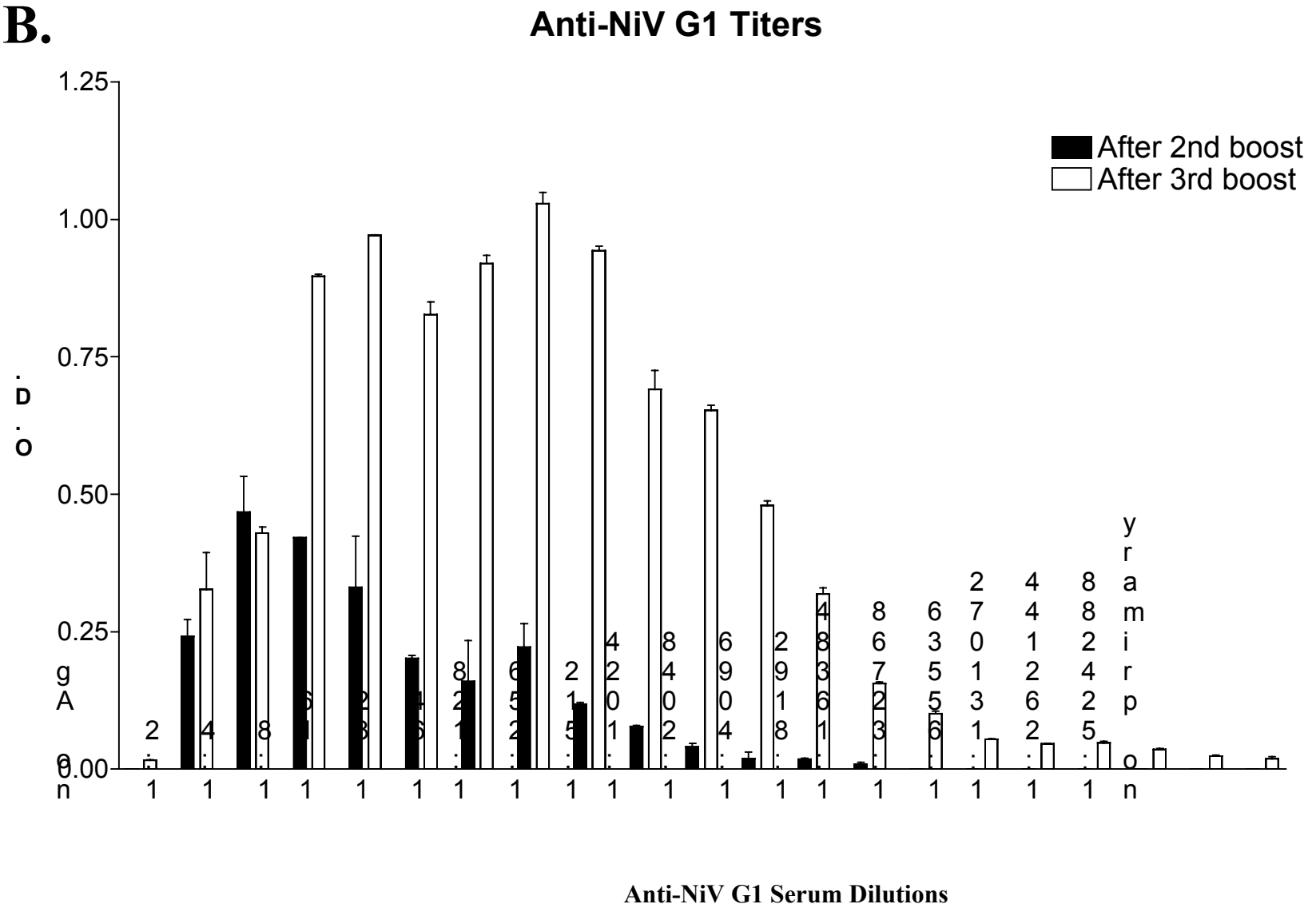
Sera Dilution

Figure 6. ELISA and Competition ELISA Results for NiV G specific serum. A. Competition ELISA. Serum was collected after the second boost and tested against the analogous peptide at different sera dilutions. The NiV G1 antiserum was diluted and either pre-mixed with 100 molar excess of analogous peptide for one hour prior to ELISA (open squares) or added straight to the ELISA plate (solid squares). In addition, the anti-F_2 and anti-G1 (HeV G) antisera were tested against the NiV G1 antigen at different dilutions to ensure specificity. No antigen and no primary antibody are internal controls for the ELISA assay. B. Serum was collected after a third boost and tested against the analogous peptide at different sera dilutions. The serum from the second bleed was included for comparison. No antigen and no primary antibody are internal controls for the ELISA assay. This experiment was done twice.

A.


Anti-NiV G1 Titers
Test Bleed
Test Bleed + Ag
0.75
0.50
0.25
0.00
Anti-NiV G1 Serum Dilutions
1:2000

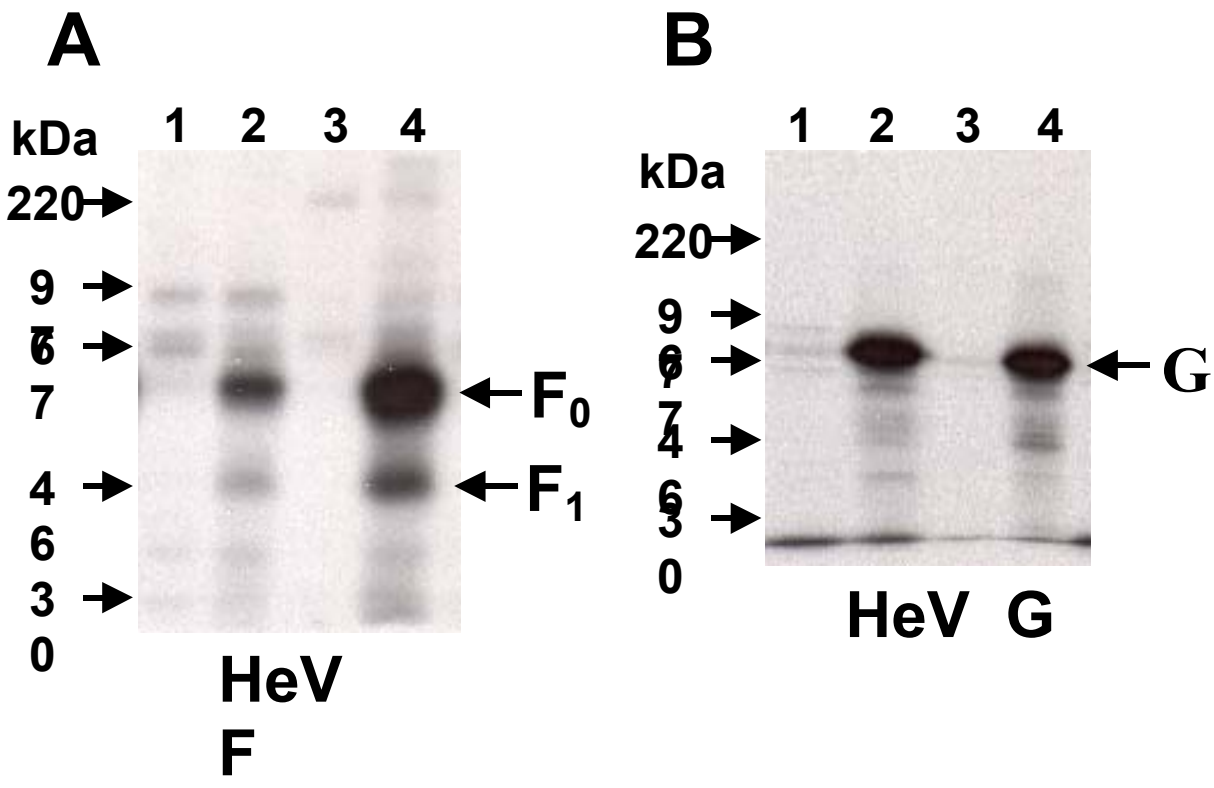
B.


Anti-NiV G1 Titers
After 2nd boost
After 3rd boost
1.25
1.00
0.75
0.50
0.25
0.00
Anti-NiV G1 Serum Dilutions

Shown in **Figure 7**, **Panel A**, is recombinant expressed HeV F immunoprecipitated with peptide-specific and virus-specific antisera. The vaccinia expressed HeV F appeared predominantly as the uncleaved precursor protein, F_0, and as the processed F_1 subunit. The F_2 subunit (~19 kDa) was not readily detected under these conditions, likely due to a combination of the amount and specific activity of the metabolically labeled polypeptide. This profile of the HeV F was quite similar to several other paramyxovirus F glycoproteins (45, 53, 59), with apparent molecular weights for F_0 of ~61 kDa and F_1 ~49 kDa, and similar to the F polypeptides derived from purified HeV particles (22, 100, 101). Shown in **Figure 7, Panel B**, is metabolically labeled recombinant expressed HeV G immunoprecipitated with rabbit anti-peptide and anti-IrHeV antisera. The vaccinia expressed HeV G exhibited an apparent molecular weight of ~75 kDa, also quite similar to HeV G derived from purified HeV virions (22, 101),as well as to the H proteins from MeV and CDV (45). The profiles of the vaccinia-expressed F and G glycoproteins observed using rabbit anti-IrHeV antiserum were identical to those obtained with the peptide-specific antisera.

Syncytia formation mediated by HeV envelope glycoproteins. To evaluate whether the F and G ORFs encoded functional HeV envelope glycoproteins, it was necessary to demonstrate their ability to mediate membrane fusion. In initial experiments, the plasmids containing either HeV F or G were transfected alone or in combination into several cell lines that included: murine 3T3, human 293T, simian BS-C-1, human TK^- and human HeLa cells. When the HeV F or HeV G constructs, or the plasmid vector

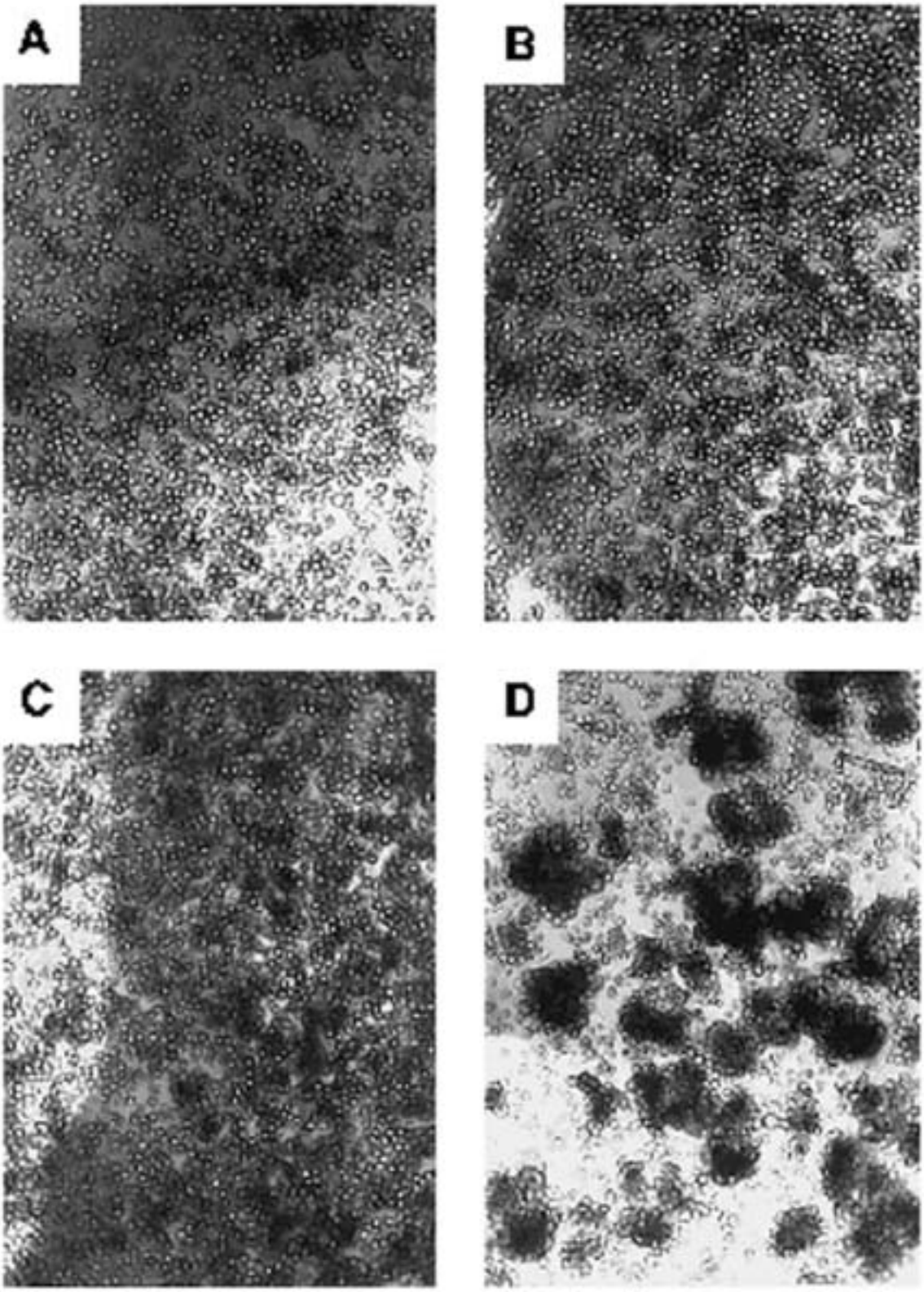
Figure 7. Expression of recombinant HeV F and G glycoproteins. The F and G glycoprotein ORFs were subcloned into a vaccinia virus promoter driven expression vector pMC02 (90). HeLa cells were infected with HeV F or G encoding constructs and incubated 16 h at 37°C. Beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine. Lysates were prepared in buffer containing Triton X-100 and clarified by centrifugation. Immunoprecipitation was performed with rabbit antisera against synthetic F or G peptides or rabbit anti-IrHeV antiserum (see Materials and Methods), followed by Protein G-Sepharose. The radiolabeled proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by fluorography. Panel A: HeV F immunoprecipitated lysates; lanes 1 and 2 with rabbit anti-IrHeV antiserum, lanes 3 and 4 with rabbit anti-F2 peptide antiserum. Panel B: HeV G immunoprecipitated lysates; lanes 1 and 2 with rabbit anti-IrHeV antiserum, lanes 3 and 4 with rabbit anti-G1 peptide antiserum. Lanes 1 and 3 of each panel are precipitates prepared from lysates of cells infected with a control vaccinia virus. This experiment was done 5 times.


A
kDa
1 2 3 4
220
97
66
7
4
6
3
0
F0
F1
HeV
F
B
kDa
1 2 3 4
220
9
6
7
4
6
3
0
G
HeV G

control were transfected into cells individually, no syncytia formation was evident in any of several cell lines examined. However, when the HeV F and G plasmid constructs were co-transfected, syncytia formation was evident in all cell lines tested with the exception of HeLa. Some variation was noted in the average size of individual syncytium among the different cell types over the same incubation period. Since HeLa cells were unable to support syncytia formation the presumption was that these cells lack a functional HeV receptor and would not serve as permissive target cells for HeV-mediated fusion. Thus, they were chosen as the HeV F and G-expressing effector cell populations for subsequent membrane fusion experiments. In so doing, HeV glycoprotein-expressing HeLa effector cells would not undergo spontaneous membrane fusion and would likely prevent possible intracellular receptor/envelope glycoprotein complex formation, which might interfere in quantitative membrane fusion assessments. Shown in **Figure 8** are the results observed with recombinant vaccinia virus-expressed HeV glycoproteins in HeLa effector cell populations mixed with murine 3T3 cells as a representative example, where syncytia is evident only when F and G are co-expressed (Figure 8, Panel D). Like most other paramyxoviruses that have been examined, HeV-mediated fusion and syncytia formation required the expression of both the F and G glycoproteins to mediate syncytia formation. These data demonstrate that the cloned ORFs for the predicted HeV F and G genes do encode functional glycoproteins.

Quantitation of HeV mediated fusion. Although syncytia formation was evident, visual observation is only semi-quantitative and possesses low sensitivity for measuring viral glycoprotein-mediated membrane fusion. Previously, a functional reporter-gene

Figure 8. Syncytia formation mediated by Hendra virus glycoprotein-expressing cells. HeLa cells were infected with vaccinia virus recombinants encoding the HeV F (Panel B), G (Panel C) or both F and G glycoproteins (Panel D), or a control vaccinia virus (Panel A), along with a vaccinia virus recombinant encoding T7 RNA polymerase. Partner 3T3 cells were infected with the *E.coli lacZ*-encoding reporter vaccinia virus vCB21R. The HeV glycoprotein-expressing cells (1×10^5) were mixed with the 3T3 partner cells (1×10^5) in duplicate wells of a 96-well plate and incubated at 37°C. After 3 h the cells were fixed and stained with crystal violet and photographs taken at 400X magnification. This experiment was done 3 times.


A
B
C
D

system for the examination of the membrane fusogenic properties of the envelope glycoprotein of HIV-1 was developed (95, 102, 103). This system is based on gene expression using the recombinant vaccinia virus system (104), where in addition to the viral envelope glycoproteins and viral receptors being expressed on effector and target cell populations respectively, one cell population also expresses bacteriophage T7 RNA polymerase and the other a T7 promoter driven E. coli. *LacZ* cassette (see Materials and Methods). Thus, cell-cell fusion results in the specific production of β-galactosidase (β-Gal), which can be quantified. **Figure 9** is a schematic diagram that illustrates this assay. This assay has proven especially useful in the study of envelope glycoproteins derived from viruses that employ a pH-independent mechanism of membrane fusion for virion entry (45, 53, 105-109). Using this assay, HeV glycoprotein-expressing HeLa effector cells were prepared and mixed with various target cell populations. Typically, the target and effector cell populations were assessed in duplicate or triplicate in 96-well plate format and incubated 2-4 hours following mixing. Cell lysates are prepared and processed for β-Gal quantification. The results shown in **Figure 10** are the HeV-mediated fusions measured in the same series of cell lines that was examined in the syncytia formation assay. Some differences in fusion activity as measured by the level of β-Gal activity were observed between those cell lines that were syncytia positive with the mouse 3T3 target cells consistently yielding the highest levels. HeLa target cells were again negative for HeV-mediated fusion, thus corroborating the syncytia assay results. Since this cell-fusion reporter gene assay is based on cytoplasmic mixing of target and effector cells, the assay is very sensitive in the detection of cell-cell fusion events and not dependent on giant cell or syncytia formation, it appears that HeLa cells are indeed fusion

Figure 9. Schematic diagram of the vaccinia based reporter gene activation assay. This system is based on gene expression using the recombinant vaccinia virus system (104), where in addition to the viral envelope glycoproteins and viral receptors being expressed on effector and target cell populations respectively, one cell population also expresses bacteriophage T7 RNA polymerase and the other a T7 promoter driven E. coli. *LacZ* cassette (see Materials and Methods). Thus, cell-cell fusion results in the specific production of β-galactosidase (β-Gal), which can be quantified**.**

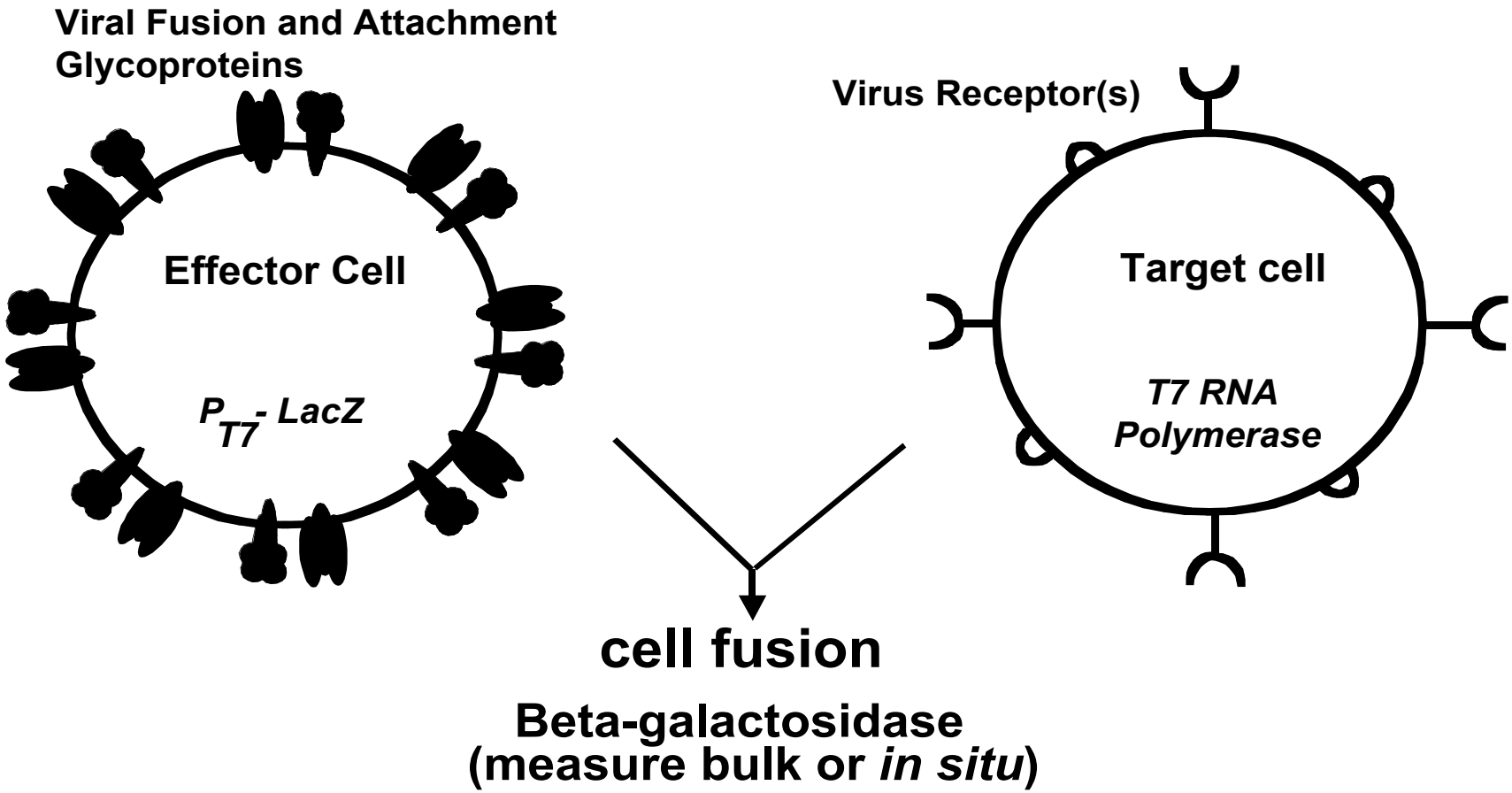
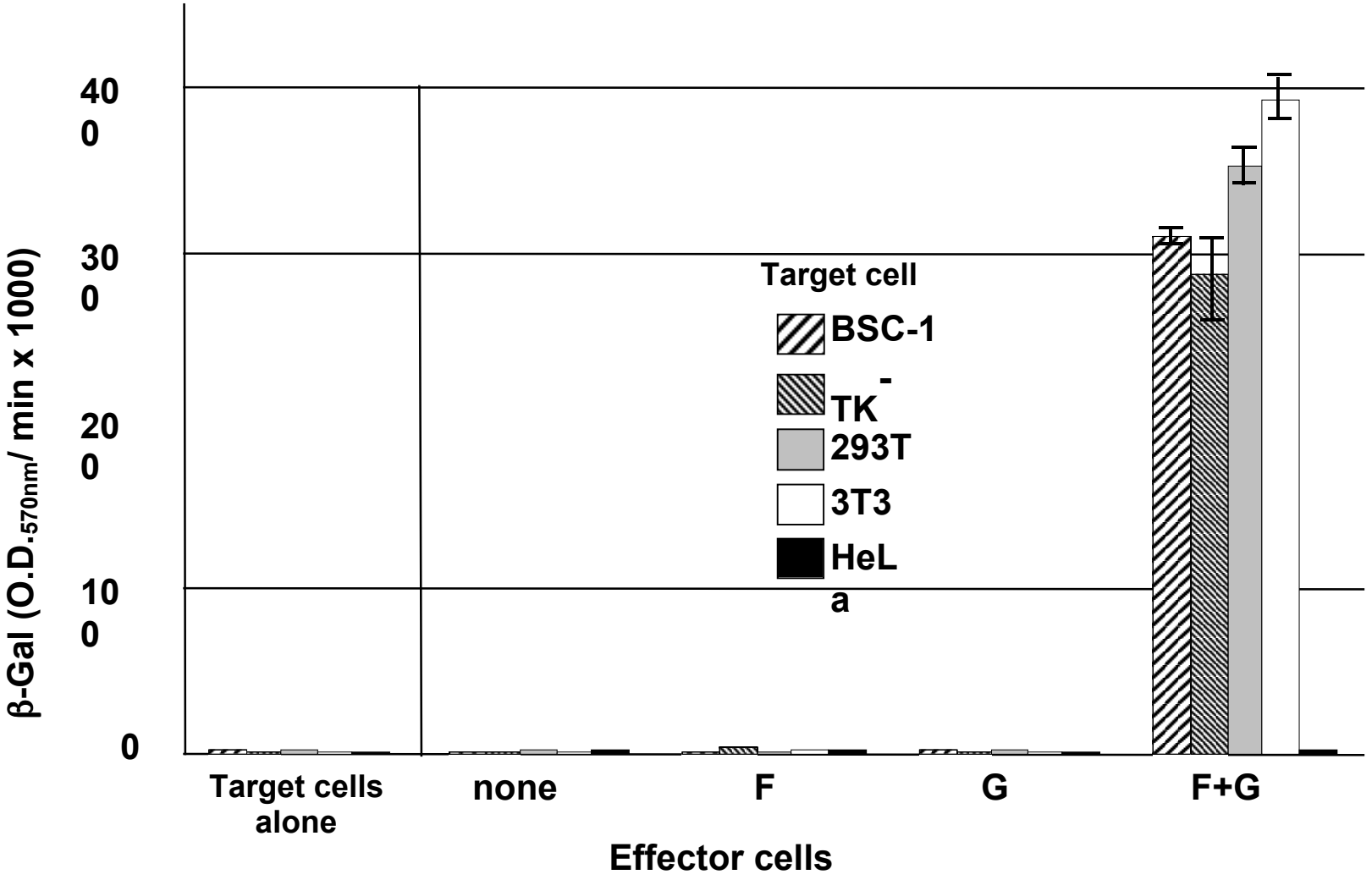

Viral Fusion and Attachment Glycoproteins
Virus Receptor(s)
Effector Cell
P_{T7}- LacZ
Target cell
T7 RNA Polymerase
cell fusion
Beta-galactosidase
(measure bulk or *in situ*)

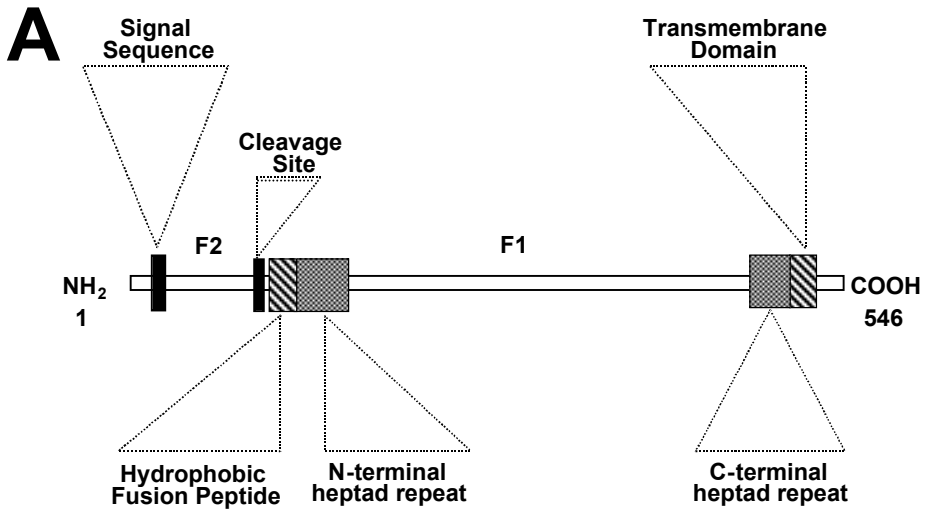
Figure 10. Quantitation of HeV mediated cell fusion. HeLa cells were infected with vaccinia recombinants encoding the HeV F, G, both F and G glycoproteins, or none, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Each designated target cell type was infected with the *E.coli lacZ*-encoding reporter vaccinia virus vCB21R. The HeV glycoprotein-expressing cells (1×10^5) were mixed with each target cell type (1×10^5) in duplicate wells of a 96-well plate. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. The level of background β-Gal activity in target cell populations alone is indicated. The β-Gal activity from target cells mixed with HeLa partner cells infected with only with T7 RNA polymerase-encoding vaccinia virus and no vaccinia recombinants encoding HeV glycoproteins is indicated as: none. This experiment was done 3 times.

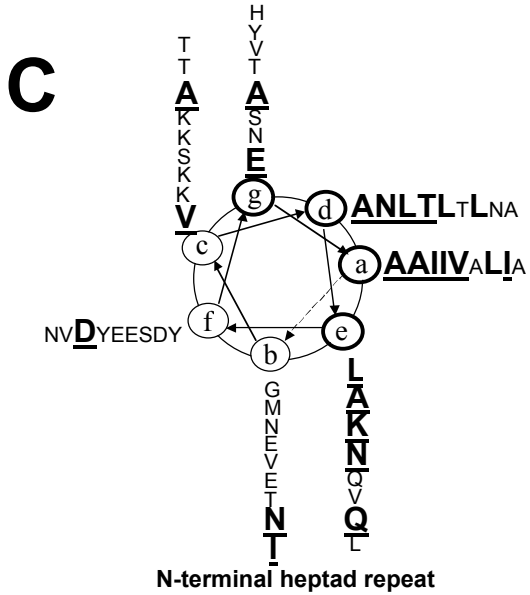

40 0
30 0
20 0
10 0
0
β-Gal (O.D.570nm/ min x 1000)
Target cell
BSC-1
TK-
293T
3T3
HeLa
Target cells alone
none
F
G
F+G
Effector cells

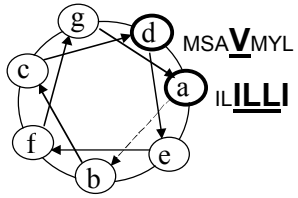
non-permissive. It is also evident from this experiment that efficient HeV-mediated membrane fusion requires the presence of both the F and G glycoproteins.

Specificity of HeV mediated fusion activity. To further assess the specificity and utility of the HeV-mediated fusion system we sought ways to specifically inhibit the cell-fusion process. There have been considerable advances in the understanding of the structural features and development of mechanistic models of how several viral envelope glycoproteins function in driving the membrane fusion reaction (reviewed in (110-112)). One important feature of many of these fusion glycoproteins are 2 α-helical domains referred to as "heptad repeats" that are involved in the formation of a "trimer-of-hairpins" structure (63, 64). These domains are also referred to as either the amino (N)-terminal and the carboxyl (C)-terminal heptad repeats, and peptides corresponding to either of these domains can inhibit the activity of the viral fusion glycoprotein when present during the fusion process. Here we noted 2 putative α-helical domains in the HeV F glycoprotein analogous to the heptad repeats present in SV5 F. One HeV F heptad domain is proximal to the fusion peptide of F_1 (N-terminal heptad repeat), and the other is very close to the predicted transmembrane domain of F_1 (C- terminal heptad repeat) (**Figure 11, Panel A**). Helical wheel analysis revealed a high degree of sequence homology with the important functional residues of the SV5 heptad repeats (**Figure 11, Panel C**) (68). To determine if these structures play a similar important role in HeV fusion, a 42 amino acid peptide (FC1) derived from the C-terminal heptad repeat (**Figure 11, Panel B**) was synthesized and tested for its ability to interfere with HeV mediated fusion. An available nonspecific 44 amino acid peptide derived from the cytoplasmic tail

Figure 11. Location of HeV F glycoprotein heptad repeats. (A) Diagram of the HeV F glycoprotein depicting important structural and functional elements. (B) Amino acid sequence of the C-terminal heptad repeat of HeV F, bold-face and enlarged amino acids represent the important hydrophobic residues that are capable of forming a leucine zipper. (C) Helical wheel representation of the N- and C-terminal heptad repeats of HeV F. Bold-faced points on the helical wheel indicate important residue locations on the helix structure of the F protein of SV5 that mediate protein-protein interactions (68). Points "a" and "d" of one N-terminal heptad are thought to interact with points "a" and "d" of another SV5 F N-terminal heptad in an antiparallel orientation. Point "e" of the N-terminal heptad is thought to interact with point "a" of the C-terminal heptad, and point "g" of the N-terminal heptad is believed to interact with point d of the C-terminal heptad. In all, it is hypothesized that three N-terminal heptad repeats and three C-terminal heptad repeats of three SV5 F proteins mediate the necessary protein-protein interactions that stabilize the fusogenic SV5 F trimer formation. Enlarged underlined amino acids represent HeV F residues that are identical to those found in the N- and C-terminal heptad repeats of SV5, enlarged but not underlined amino acids are hydrophobic conservative substitutions in HeV F as compared to SV5 F.







B

SIAVGPPVYTDKVDISSQISSMNQSLQQSKDYIKEAQKILD
TVNPSL **C-terminal heptad repeat sequence (442-489)**

of the IL-2 receptor gamma chain was used as an irrelevant peptide control. Shown in **Figure 12**, are the results of HeV-mediated fusion obtained in the presence of a series of FC1 peptide, or irrelevant control peptide, concentrations. The FC1 peptide could potently inhibit fusion in a dose-dependent manner, and was completely inhibitory in the nM range. These data strongly suggest that the HeV fusion mechanism is likely highly analogous to other viral fusion systems where a trimer of hairpins has been hypothesized to form, and the specific inhibition of the HeV-mediated fusion assay by a synthetic peptide which targets the F glycoprotein further demonstrates the specificity of this HeV-mediated cell fusion assay.

To explore the utility of the HeV fusion assay, we tested the ability of HeV-specific antiserum to inhibit fusion. Rabbit anti-IrHeV antiserum was serially diluted and added to HeLa effector cells expressing HeV F and G glycoproteins. Target cells were immediately added and mixed, and fusion was allowed to proceed for 3 h (see Materials and Methods). Shown in **Figure 13**, are the HeV fusion results obtained in the presence of HeV-specific rabbit antiserum in comparison to either no or normal rabbit serum. There was little non-specific inhibitory activity exhibited by the normal rabbit serum with the maximal activity ~15%, at the highest sera concentrations. Inhibition by the HeV-specific antiserum could block the fusion assay by 90% at a 1:50 dilution and there was approximately 50% inhibition at a 1:200 dilution. These results were quite significant in light of the high levels of expressed HeV F and G envelope glycoproteins on the surfaces of the HeLa effector cells, and the potency of inhibition observed here may be related to the requirement of two envelope glycoproteins in the HeV fusion process.

Figure 12. Specificity of HeV-mediated fusion. HeLa cells were infected with vaccinia recombinants encoding the HeV F and G glycoproteins along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Human TK^- cells were infected with the *E.coli lacZ*-encoding reporter vaccinia virus vCB21R (target cells). Peptides were diluted and added to the HeV glycoprotein-expressing cells (1×10^5) in a 96-well plate, TK^- cells were then added (1×10^5). Each peptide concentration was performed in duplicate in 96-well plate format. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. This experiment was done twice.

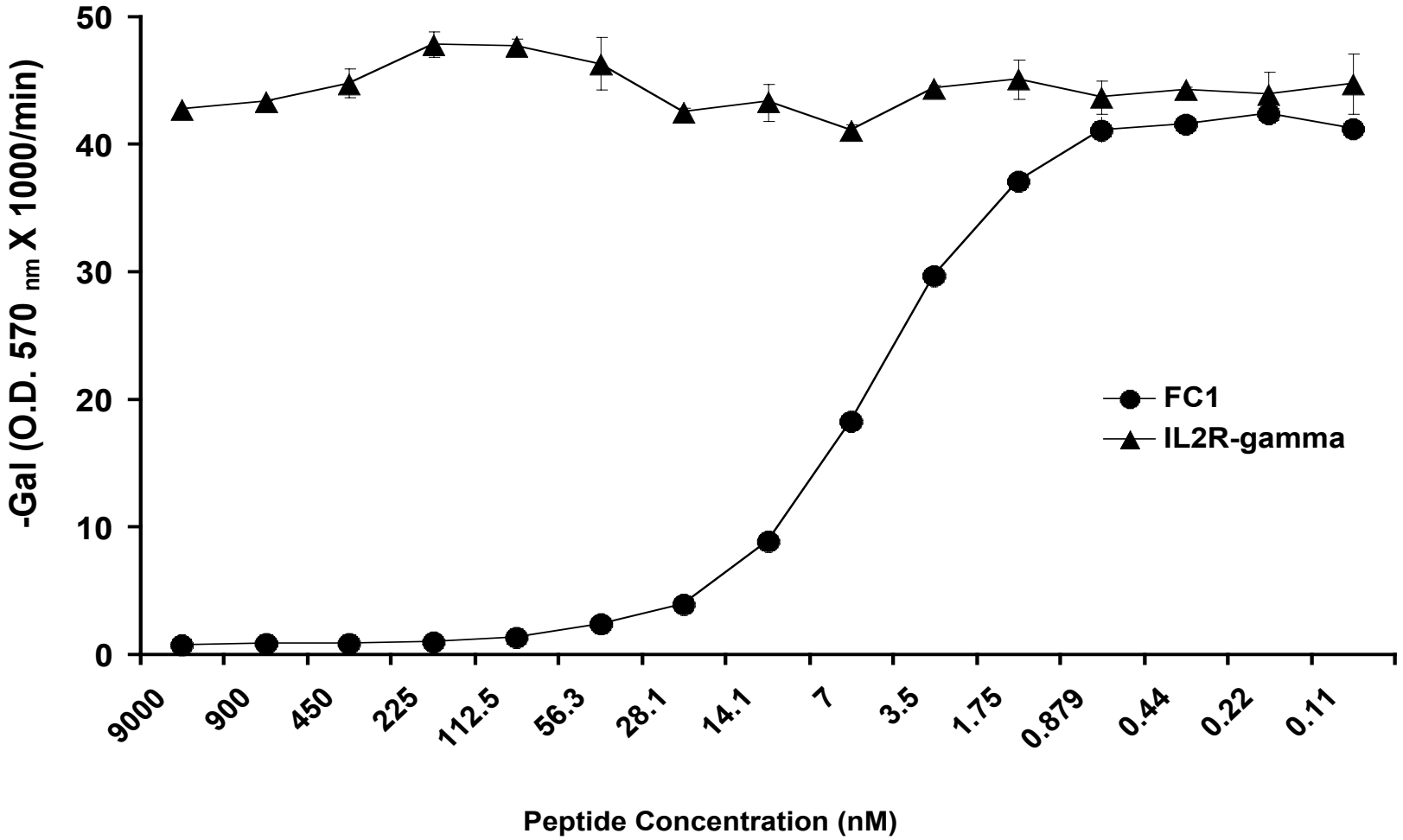
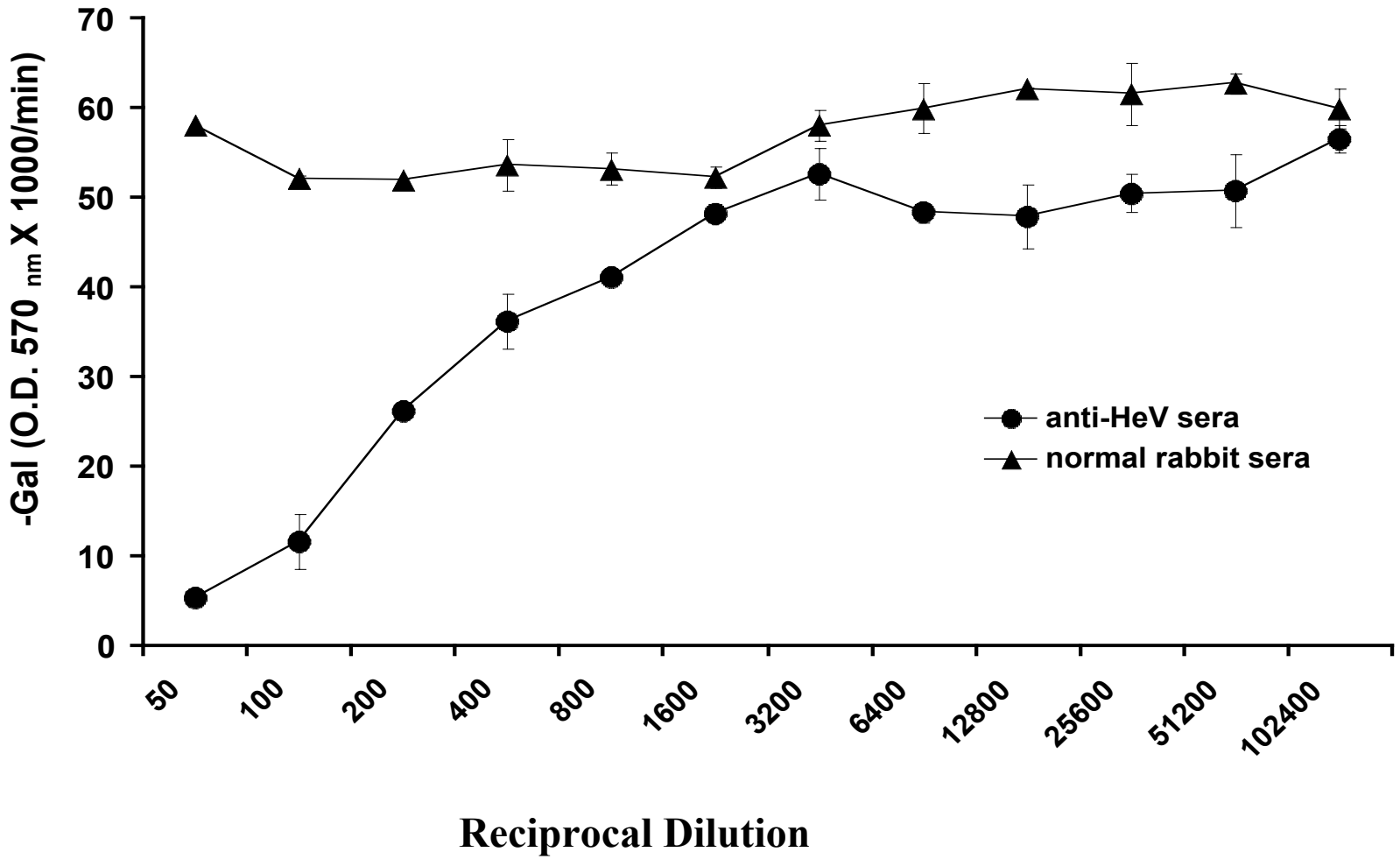

-Gal (O.D. 570 nm X 1000/min)
50
40
30
20
10
0
9000
900
450
225
112.5
56.3
28.1
14.1
7
3.5
1.75
0.879
0.44
0.22
0.11
Peptide Concentration (nM)
FC1
IL2R-gamma

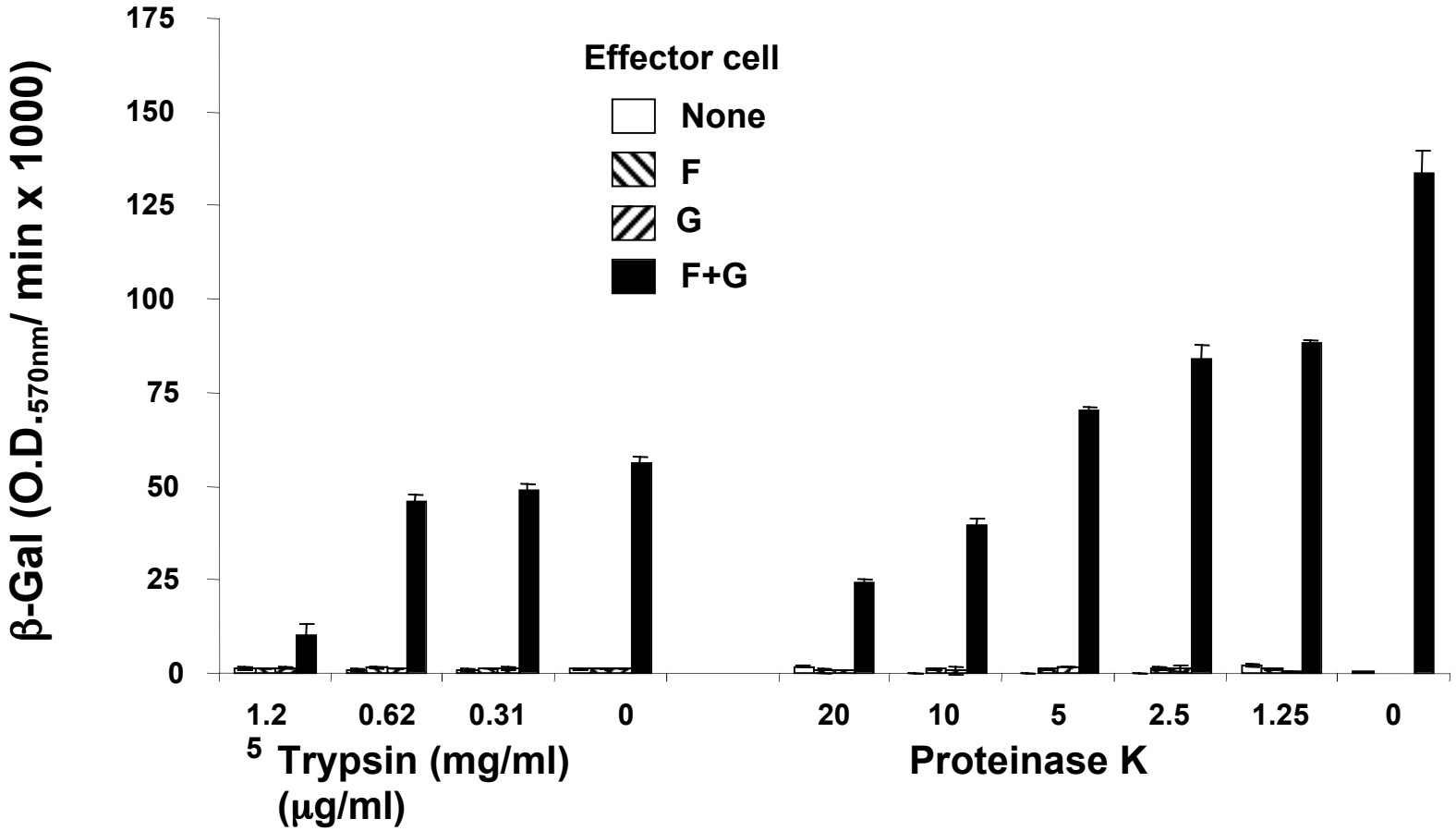
Figure 13. HeV fusion and blocking by specific rabbit antiserum. HeLa and TK^- cells were prepared as described in the legend of Figure 12. Rabbit sera were diluted and added to the HeV glycoprotein-expressing cells (1×10^5) in 96-well plate format and TK^- target cells were then added (1×10^5). Each sera dilution was performed in duplicate wells. After 3 h at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. This experiment was done twice.


70
60
50
40
30
20
10
0
-Gal (O.D. 570 nm X 1000/min)
anti-HeV sera
normal rabbit sera
50
100
200
400
800
1600
3200
6400
12800
25600
51200
102400
Reciprocal Dilution

Protease treatment of target cells destroys HeV-mediated fusion activity. The utility of the HeV-mediated fusion assay combined with the observation that cell lines derived from the same species may be either permissive or resistant to fusion prompted an examination of the nature of the unknown HeV receptor, and permissive target and effector cells were subjected to various pre-treatments prior to their use in the cell fusion assay. HeV does not exhibit hemagglutinating or neuraminidase activity (23, 113) and, thus, the cellular receptor may not be sialic acid. We treated effector cells with excess sialic acid over a range of concentrations and in no case was any inhibitory effect observed on HeV-mediated fusion (data not shown). We have also shown that neuraminidase treatment of Vero cells does not inhibit HeV infection but can abrogate their susceptibility to NDV and influenza virus A, two viruses which employ sialic acid as receptors (Eaton, B.T. unpublished). Together, these data are in agreement with the notion that the HeV G protein is not employing sialic acid moieties as receptors. However, protease treatment of target cells with increasing doses of either proteinase K or trypsin, resulted in significant decreases in subsequent HeV-mediated fusion in a dose-dependent manner (**Figure 14**). Together, these results support the hypothesis that HeV, like one of its most closely related family members, MeV, is likely employing a surface-expressed cellular protein as a functional receptor for attachment and fusion.

Species tropism of HeV-mediated cell fusion. Unlike other paramyxoviruses, HeV has been clearly implicated in cross species infections, including human, that can result in significant morbidity and mortality. Using the HeV-mediated cell fusion system developed here, we examined the target cell species tropism of HeV using a battery of

Figure 14. Protease treatment of target cells destroys HeV-mediated fusion permissiveness. HeLa and TK^- cells were prepared as described in the legend Figure 12. TK^- target cells were treated with different concentrations of either trypsin or proteinase K for 2 minutes at room temperature, quenched with 10 ml of EMEM-10, washed once, and recounted. HeV glycoprotein-expressing cells (1×10^5) and protease treated TK^- cells (1×10^5) were then mixed in 96-well plate format in duplicate. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. The trypsin and Proteinase K treatment experiments were completed independently and the data was merged into one figure. These experiments were completed once.


β-Gal (O.D.570nm/ min x 1000)
175
150
125
100
75
50
25
0
Effector cell
None
F
G
F+G
1.2
0.62
0.31
0
20
10
5
2.5
1.25
0
5 Trypsin (mg/ml)
(μg/ml)
Proteinase K

available cell lines and primary cultures. Shown in **Figure 15** are the cell fusion results obtained with a series of alternate animal species cell lines. Target cells derived from an insectivorous bat, horse (the first animal to contract HeV disease), and human TK^- cells were all capable of permissive fusion with cells expressing the HeV F and G glycoproteins. Cat embryo cells were also permissive targets for HeV-mediated fusion, and cats have been shown to be highly susceptible to HeV infection, manifesting pathology very similar to that observed in naturally and experimentally infected horses (86). These results illustrate that the cell fusion tropism demonstrated by our functional recombinant assay parallels natural and experimental HeV infections. Other cells that were negative for fusion were a pig kidney and duck embryo cell lines, as well as primary chick embryo fibroblasts. Although rabbits, monkeys, and mice have not been shown to be infected by HeV in nature, it is of interest that these cells also express the HeV receptor. The susceptibility of rabbits to HeV infection remains to be verified. In all cases, both HeV F and G together were required to mediate fusion with all permissive target cell populations (**Figure 15**). Finally, because both permissive and non-permissive human target cell lines have been identified, we chose to examine primary blood cell populations of human peripheral blood lymphocytes (PBL) and macrophages. Both stimulated and unstimulated PBL and macrophages were examined, and compared to HeLa target cells, however in no case was significant fusion observed (**Figure 16**). It should be noted that the level of signal observed is extremely low and that the signal observed with HeLa cell targets is less than twice the vector control and not considered significant. The hypothesis that HeV utilizes a cellular protein receptor in the process leading to fusion and syncytium formation and the fact that HeLa cells lack this receptor or at least a functional receptor,

Figure 15. Species tropism of HeV-mediated cell fusion. HeLa effector cells were prepared as described in the legend of Figure 10. Each designated target cell type was infected with the *E.coli lacZ*-encoding reporter vaccinia virus vCB21R. Cell populations were mixed and cell fusion was measured as described in the legend of Figure 10. This experiment was done twice.

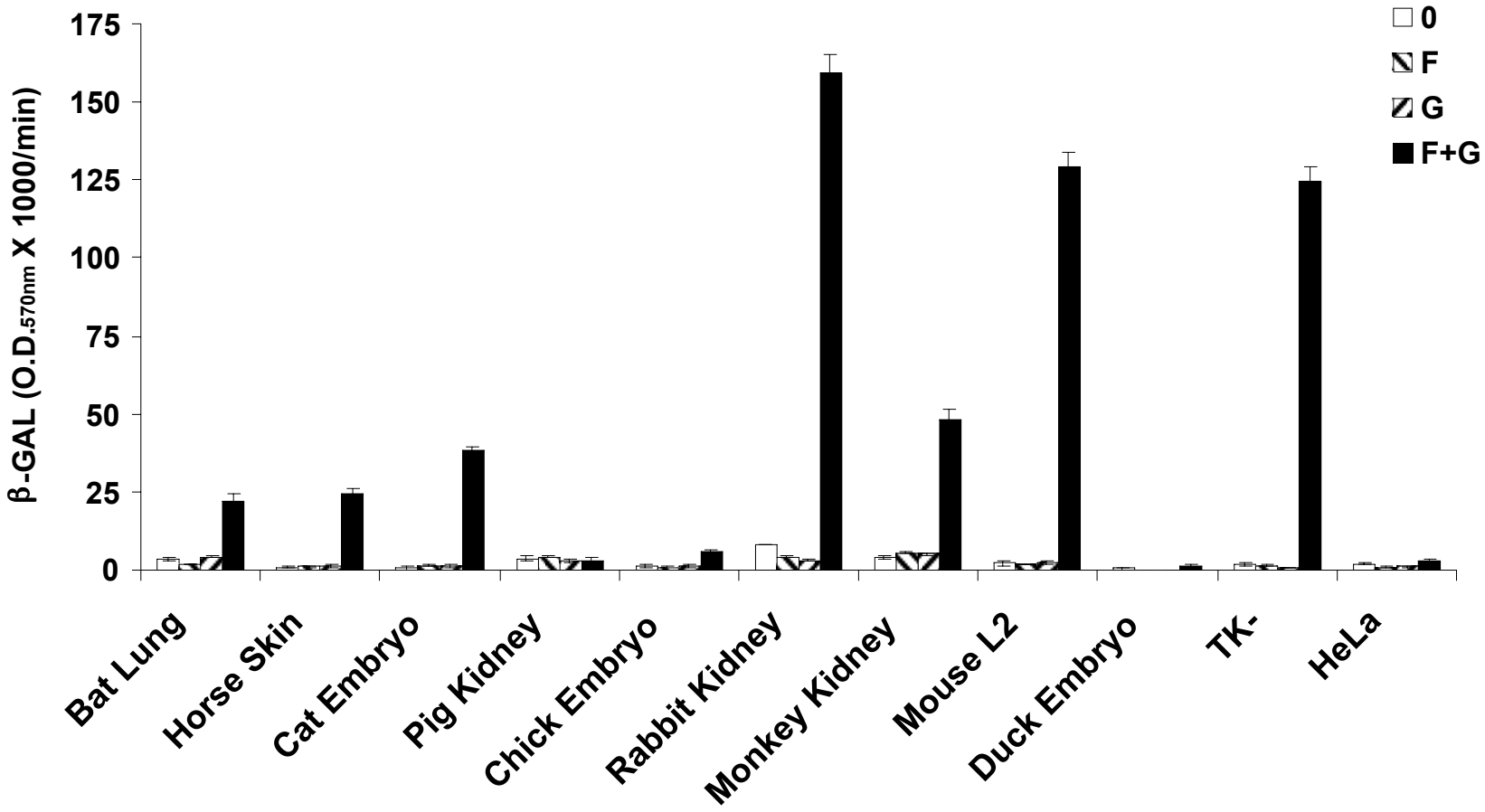
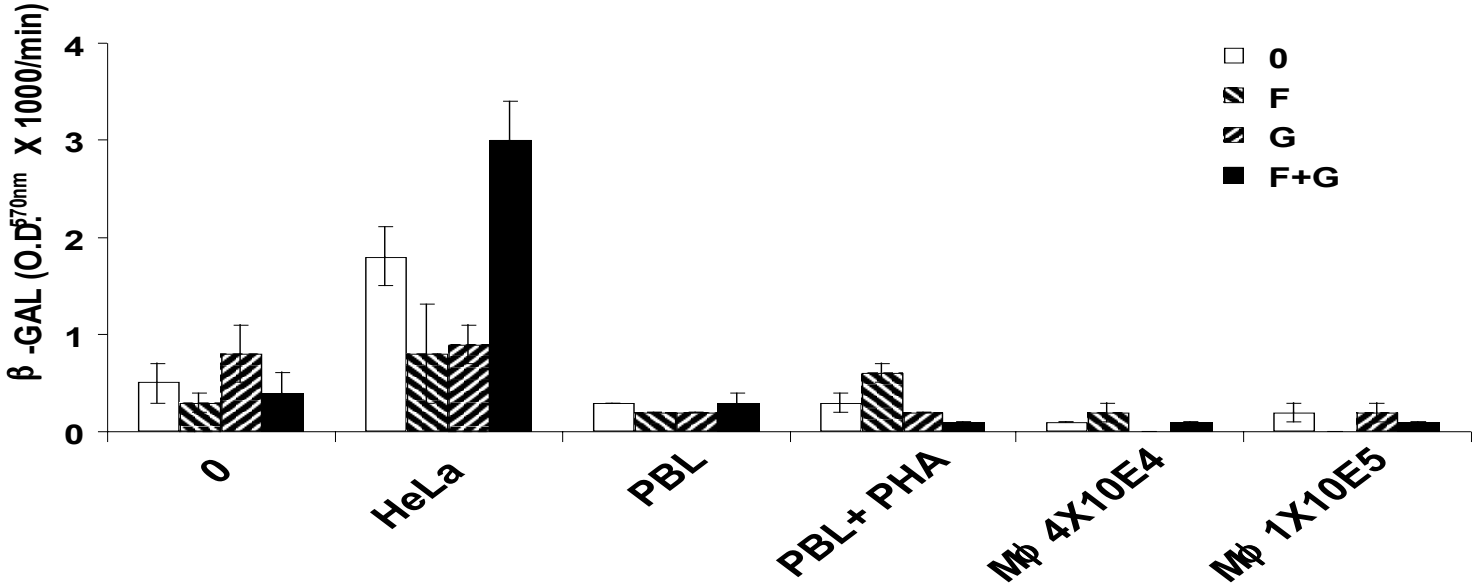

β-GAL (O.D.570nm X 1000/min)
175
150
125
100
75
50
25
0
0
F
G
F+G
Bat Lung
Horse Skin
Cat Embryo
Pig Kidney
Chick Embryo
Rabbit Kidney
Monkey Kidney
Mouse L2
Duck Embryo
TK-
HeLa

Figure 16. Primary human cell tropism of HeV-mediated cell fusion. Human PBL and macrophage cultures were incubated overnight prior to use. Stimulated PBL were prepared by culturing cells with 3μg/ml PHA for 3 days prior to use. HeLa effector cells were prepared as described in the legend of Figure 10. Each designated target cell type was infected with the *E.coli lacZ*-encoding reporter vaccinia virus vCB21R. Cell populations were mixed and cell fusion was measured as described in the legend of Figure 10. This experiment was done at the same time as the tropism data shown in Figure 15, however, the target cells shown in Figure 16 were only tested once.


β -GAL (O.D.570nm X 1000/min)
4
3
2
1
0
0
F
G
F+G
0
HeLa
PBL
PBL+ PHA
Mφ 4X10E4
Mφ 1X10E5

coupled with this highly sensitive and specific HeV-mediated fusion system, provides an avenue for receptor identification.

DISCUSSION

The results presented here have laid the groundwork for studying the fusion and attachment membrane glycoproteins of the newly emerging Hendra virus. To date, the genetic and biological characterizations of HeV, as well as the related Nipah virus (NiV), have indicated the necessity of creating a new genus (*Henipavirus)* in the *Paramyxovirinae* subfamily (16, 18, 32, 44, 86). Indeed, HeV and NiV are somewhat unique among the paramyxoviruses in their ability to cause severe and fatal disease in several animal species and humans. Understanding the mechanisms of how viruses like these can emerge, mediate host cell infection and cross species is an important step towards determining how to address new infectious disease threats such as these.

Here we report the development of a recombinant expression system to examine the membrane glycoproteins of HeV and describe several features of their functional activity. Similar results have been recently obtained in our laboratory with the membrane glycoproteins of NiV (Bossart and Broder, unpublished). Radioimmunoprecipitation using anti-HeV peptide-specific sera and rabbit anti-IrHeV antiserum showed that recombinant vaccinia virus-expressed HeV F and G glycoproteins were comparable to the cognate proteins in purified virus (101). The molecular weight of the F_0 precursor was ~61 kDa and the processed F_1 subunit ~49 kDa. The F_2 subunit, ~19 kDa, was not observed biochemically using our reagents. The failure of F_2 detection by either antisera could be due to the paucity of F_2 antibodies, but is more likely related to assay sensitivity. Nevertheless, F2 is present based on the functionality of HeV F glycoprotein in fusion and the fact that the anti-peptide antiserum used is specific for an F_2 sequence. In addition, we have observed a similar lack of F_2 detection in radioimmunoprecipitation of functional

MeV and CDV F glycoproteins (45). The recombinant expressed G glycoprotein was ~75 kDa. Both these glycoproteins have several predicted N-linked glycosylation sites (100) and studies are underway to examine which sites are utilized and are important for biological function. Based on the similarity in molecular weights in comparison to other members of the Paramyxoviridae the F and G glycoproteins of HeV are undoubtedly N-glycosylated at one or more sites.

The functionality of the HeV F and G glycoproteins was immediately apparent through recombinant expression and the appearance of syncytia among cell populations expressing both HeV F and G glycoproteins. Indeed, the HeV glycoproteins were functionally expressed in pilot experiments prior to the availability of antisera for biochemical detection. By adapting a cell-cell fusion reporter-gene assay to the HeV-mediated fusion system we were able to examine systematically, in a quantitative manner, a battery of target cell populations representing a variety of cell types and animal species. In so doing, and more importantly for the development of strategies to identify the receptor utilized by HeV, several cell lines and some primary cell types were found to be likely receptor-negative for HeV. Among these possible receptor-negative cell types were the human HeLa cell line and primary human PBLs and macrophages. It was somewhat surprising that pig cells were negative, in light of the observation that pigs are natural hosts of the related NiV in nature. The examination of additional pig cell lines and or primary cells will be undertaken when they become available. In general however, the detection of HeV-mediated fusion correlated well with those animal species known to be permissive for experimental HeV infection, such as horse, cat and, bat. Although we examined an insectivorous bat cell line, the natural reservoir of HeV appears to be a frugiverous bat.

The generation of very high titers of anti-IrHeV antibodies following administration of a single dose of HeV to rabbits suggests that such animals may be susceptible (114) and the susceptibility of rabbit kidney cells to fusion is consistent with this suggestion. However, some caution must be exercised in correlating *in vivo* susceptibility to infection with ability to form syncytia in an in vitro system. The capacity of mouse L2 cells to form syncytia contrasts with the failure of HeV to infect BALB/c mice by intranasal or parental routes (Eaton, B.T. unpublished).

To date, MeV, a morbillivirus and one of the most closely related viruses to HeV and NiV, is the only paramyxovirus shown capable of employing a cell-surface protein as a functional receptor: CD46 (115), and the MeV attachment glycoprotein: hemagglutinin (H) has been shown to specifically complex with CD46 (45). Here we assessed the nature of the unknown HeV cellular receptor using the cell-cell fusion assay and found that protease treatment of permissive target cells inhibits HeV-mediated membrane fusion. Similar experiments in the characterization of HIV-1 envelope-mediated cell-cell fusion had also demonstrated this inhibition, where the CD4 receptor is readily removed from the cell surface by trypsin (116). We hypothesize that HeV is likely to utilize a surface expressed protein receptor for virus entry and infection. We speculate this because the HeV attachment glycoprotein does not have hemagglutinin or neuraminidase activities, protease treatment prevents fusion of an otherwise permissive target cell, and certain cell lines from the same species can be clearly positive or negative for fusion. The cell-cell fusion system described here is ideally suited for use in an expression cloning strategy for identifying the HeV receptor as was successfully done to discover the first HIV-1 co-receptor (117).

All viral membrane glycoproteins that are the mediators of membrane fusion, virion attachment or both, are invariably oligomeric (73). Considerable advances in the understanding of the structural features of these oligomeric viral envelope glycoproteins has been attained in recent years and have centered on the influenza virus and HIV systems. A notable structural feature of many of these fusion glycoproteins is the presence of 2 α-helical domains referred to as heptad repeats that are important for both oligomerization and function of the glycoprotein, where they are involved in the formation of a "trimer-of-hairpins" structure (63, 64). Peptides corresponding to either of these domains can potently inhibit the fusion process, first noted with sequences derived from the gp41 subunit of HIV-1 envelope glycoprotein (65, 66). Inhibition of the formation of the trimer-of-hairpins structure inhibits the fusion process, and this mechanism has been modeled and described by several groups (118-121). Indeed, the development and clinical application of fusion-inhibitors, as antiviral therapies for HIV-1, has been a direct result from this area of research.

Recently, an α-helical trimeric core complex has been defined in the F protein of SV5 and it is also believed to be either the fusion competent structure or the structure formed after fusion has occurred, analogous to HIV-1 gp41 (122). In addition, peptide sequences from the C-terminal heptad of SV5 F, as well as MeV F, have been shown to be potent inhibitors of membrane fusion (68). Here we analyzed the heptad repeats of HeV F using helical wheel diagrams and identified the sequences that would be likely inhibitors of HeV-mediated fusion. We then examined the specificity of our recombinant HeV fusion system using a synthetic 42 amino acid peptide (FC1) corresponding to the HeV F C-terminal heptad. The FC1 peptide could completely inhibit HeV-mediated fusion in the

nM range. It is presently being evaluated as an inhibitor of live HeV infection under BSL-4 conditions, and may represent a therapeutic avenue for both HeV and NiV infections. Indeed, the HeV F C-terminal heptad peptide was also capable of inhibiting recombinant NiV-mediated fusion at slightly lower efficiencies; this is likely due to several mismatches in the heptad sequence (Bossart and Broder, unpublished).

HeV cell-cell fusion was also characterized using HeV-specific rabbit antiserum that could block the cell-cell fusion assay with considerable efficiency. In addition, NiV-specific rabbit serum could also block HeV-mediated fusion at lower levels of efficiency (not shown) and this would be in agreement with the observed antigenic cross-reactivity seen with the HeV and NiV (123). Because this assay can be performed under BSL-2 conditions, and is highly adaptable to high-throughput screening, it may be a useful tool in the titering of neutralizing antisera outside BSL-4 containment. Experiments are underway to make comparative assessments of this cell-cell fusion assay with live HeV neutralization assays.

In summary, we have established a recombinant system to express and characterize the HeV F and G membrane glycoproteins and study the HeV-mediated membrane fusion process. We have shown that efficient membrane fusion requires both the F and G glycoproteins as is seen for almost all other paramyxoviruses. In addition, fusion can be specifically inhibited with either antiserum or targeted peptides, and to a significant degree fusion parallels observed and experimental HeV infection. In addition, we have identified possible receptor-negative cell types. These preliminary studies have laid the foundation for numerous approaches to develop new reagents and to examine the

many features of the fusion and attachment glycoproteins of this interesting and unique emerging paramyxovirus.

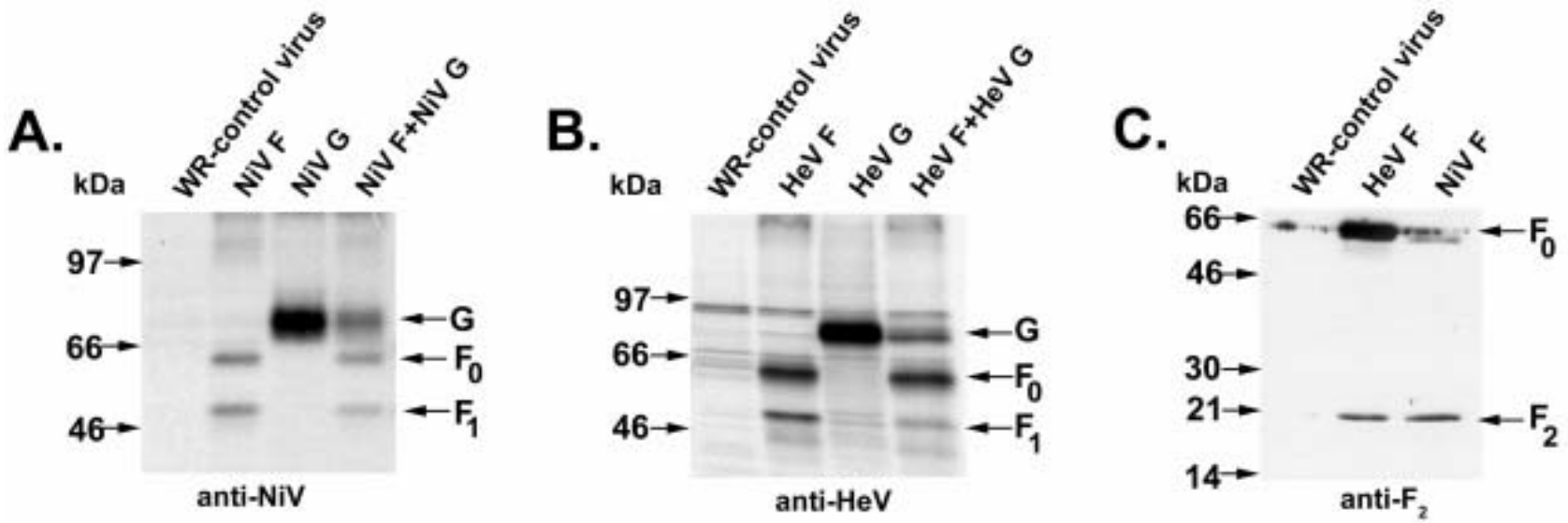
Chapter 4

Membrane Fusion Tropism and Heterotypic Functional Activities of the Nipah Virus and Hendra Virus Envelope Glycoproteins.

RESULTS

Expression of Henipavirus F and G glycoproteins. To examine the functional and biochemical properties of the NiV and HeV envelope glycoproteins, the proteins responsible for host cell attachment and virus entry, we have employed the vaccinia virus-based recombinant expression system. Recently we described this system to study the envelope glycoproteins of HeV (124). For the production of recombinant-expressed NiV envelope glycoproteins, the putative glycoprotein open reading frames for NiV F and G were subcloned into the vaccinia virus promoter driven expression vector pMCO2 (90) and recombinant vaccinia viruses were generated using standard techniques as detailed in the Materials and Methods. NiV envelope glycoproteins F and G were produced in cell culture by infection with recombinant vaccinia viruses. Shown in **Figure 17 A** are immunoprecipitation results for recombinant vaccinia virus-expressed NiV F, G, or both F and G, using NiV- or HeV-specific antisera. Vaccinia-expressed NiV F appeared as the precursor protein, F_0, and as the processed F_1 subunit. The F_2 subunit (~19 kDa) was not readily detected under these conditions, most likely owing to a combination of the amount of protein and the specific activity of the metabolically labeled polypeptide. This profile of the NiV F was quite similar to recombinant expressed HeV F (124) which is also shown for comparison (**Figure 17B**), as well as to several other paramyxovirus F glycoproteins (45, 53, 59), with apparent molecular

Figure 17. Expression of recombinant NiV F and G glycoproteins. The NiV F and G glycoprotein ORFs were subcloned into a vaccinia virus promoter driven expression vector pMC02 (90), and recombinant viruses were made (see Materials and Methods). HeLa cells were infected with NiV F or G encoding viruses and incubated 16 h at 37°C. Beginning at 6 h post-infection, the cells were either labeled overnight with [^{35}S]-methionine/cysteine for immunoprecipitation or cultured in medium alone for western blotting. Lysates were prepared in buffer containing Triton X-100 and clarified by centrifugation. Immunoprecipitation was performed with rabbit anti-IrNiV or rabbit anti-IrHeV antisera followed by Protein G-Sepharose. Western Blot was performed using rabbit antiserum against a synthetic F_2 peptide (see Materials and Methods). The metabolically labeled proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by fluorography; lysates for Western blot were resolved by 10% SDS-PAGE under reducing conditions and detected by chemiluminescence. Panel A: Immunoprecipitation; lane 1: WR-control vaccinia virus; lane 2: recombinant expressed NiV F; lane 3: recombinant expressed NiV G; lane 4: recombinant expressed NiV F+G. Panel B: Immunoprecipitation; lane 1: WR-control vaccinia virus; lane 2: recombinant expressed HeV F; lane 3: recombinant expressed HeV G; lane 4: recombinant expressed HeV F+G. Panel C: Western blot; lane 1: WR; lane 2: recombinant expressed HeV F; lane 3: recombinant expressed NiV F. This experiment was done 5 times.


A.
kDa
WR-control virus
NiV F
NiV G
NiV F+NiV G
97
66
46
G
F0
F1
anti-NiV
B.
kDa
WR-control virus
HeV F
HeV G
HeV F+HeV G
97
66
46
G
F0
F1
anti-HeV
C.
kDa
WR-control virus
HeV F
NiV F
66
46
30
21
14
F0
F2
anti-F2

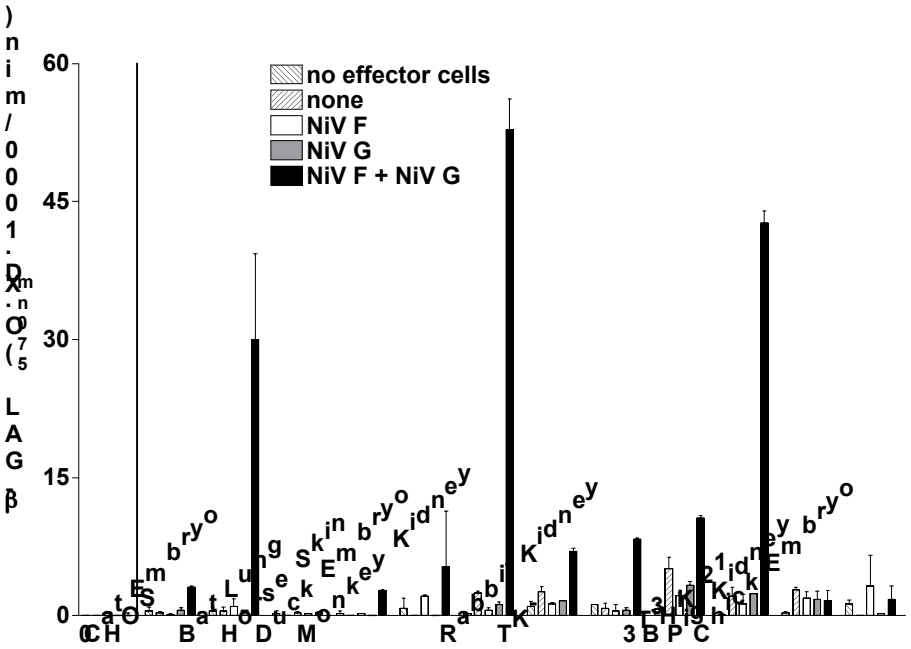
weights for F_0 of ~61 kDa and F_1 ~49 kDa, and similar also to the F polypeptides derived from purified HeV particles (22, 100, 101). The recombinant vaccinia-expressed NiV G possessed an apparent molecular weight of ~75 kDa, also quite similar to recombinant vaccinia-expressed HeV G (**Figure 17B**) (124) and HeV G derived from purified HeV virions (22, 101), as well as to the H proteins from MeV and CDV (45). Shown in **Figure 17C**, is recombinant vaccinia virus-expressed NiV and HeV F detected by Western blot using HeV F_2 peptide-specific antiserum. NiV F appears to be more readily processed than HeV F as determined by a marked reduction of the NiV F_0 species. F_0 and F_2 for both HeV and NiV migrate close to their predicted molecular weight of ~61 kDa and ~19 kDa., respectively.

Membrane fusion tropism mediated by HeV and NiV F and G glycoproteins. The adaptation of a previously developed reporter-gene assay that is capable of quantitative measurement of cell-fusion mediated by the viral envelope glycoproteins of both HeV and NiV has afforded several avenues of investigating the nature of these otherwise BSL-4 restricted agents. This system is based on gene expression using the recombinant vaccinia virus system (95, 96), where, in addition to the viral envelope glycoproteins and viral receptors being expressed on effector and target cell populations, respectively, one cell population also expresses bacteriophage T7 RNA polymerase and the other a T7 promoter driven *E. coli. LacZ* cassette (see Materials and Methods). Thus, cell-fusion results in the specific production of β-galactosidase (β-Gal), which can be quantified. This assay has proven especially useful in the study of envelope glycoproteins derived from viruses that employ a pH-independent mechanism of membrane fusion for virion

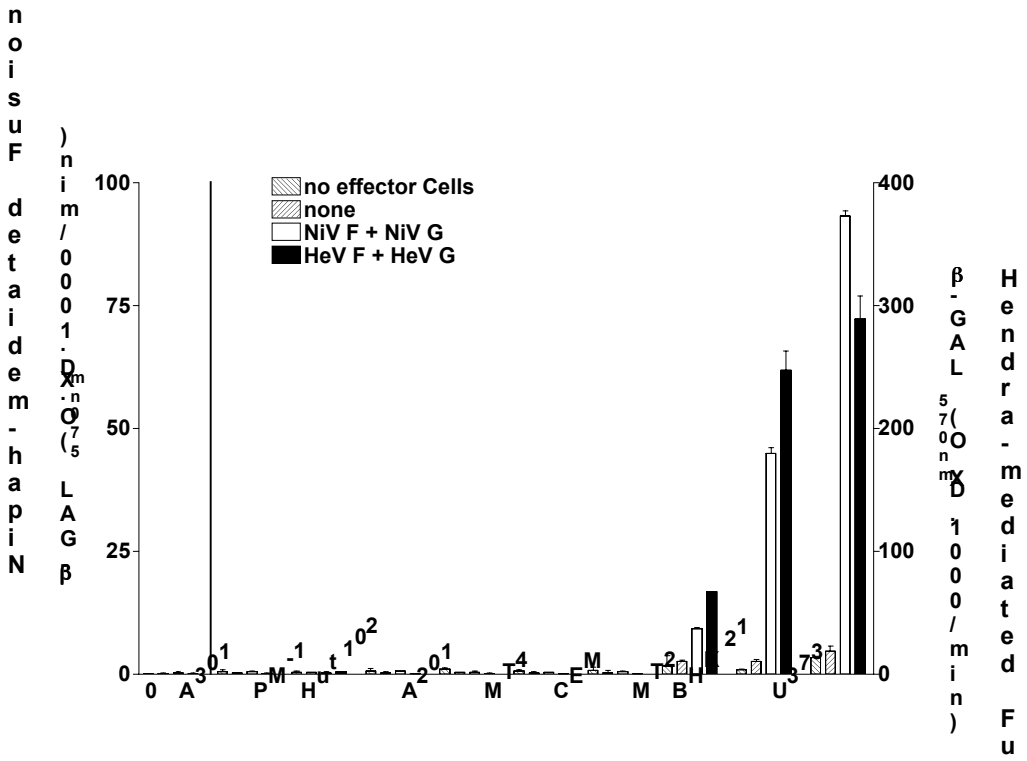
entry (45, 53, 105-109, 117). Using this assay, NiV glycoprotein-expressing effector cells were prepared and mixed with various target cell populations. Typically, the target and effector cell populations are assessed in duplicate or triplicate in 96-well plate format and incubated 2-4 hours following mixing. Cell lysates are prepared and processed for β-Gal quantification. Initial experiments using HeLa cells for NiV F and G expression (effector cells) and BSC-1 and HeLa cells as putative receptor positive cells (target cells) rapidly revealed that like HeV, HeLa cells were non-permissive for NiV-mediated fusion and that NiV requires both the F and G envelope glycoproteins to mediate fusion with receptor positive cell lines (data not shown). Since HeLa cells were not permissive for NiV-mediated fusion, they were therefore selected for expression of NiV F and G expression in subsequent experiments (effector cells). The evaluation of host cell tropism by measuring cell-fusion was then expanded to include a variety of target cells, including those previously examined for their ability to support HeV-mediated fusion. **Figure 18A** illustrates that both NiV F and G are needed to mediate cell-fusion, and a that wide panel of cell lines from a variety of animal species appear to have the NiV receptor on their cell surface. Shown in **Figure 18B** are HeV and NiV-mediated fusion results with additional human T-cell lines and U373, a human glioblastoma cell line, as target cells. The MT2 cell line is the first T cell line examined that appears to express the HeV and NiV receptor. These data also demonstrated that the U373 cell line supported the highest level of NiV-mediated cell-fusion, which may reflect the neural tropism of the virus and subsequent pathology seen in Nipah-infected humans and animals (125-127). For these reasons, the U373 cells were included as an important target cell in subsequent experiments. Although HeV fusion rates were not shown **in Figure 18A**, NiV F and G

Figure 18. Quantitation of NiV-mediated cell fusion. HeLa cells were infected with vaccinia recombinants encoding NiV F, G, both NiV F and G glycoproteins, neither (none), or both HeV F and G, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Each designated target cell type was infected with the *E.coli LacZ*-encoding reporter vaccinia virus vCB21R. The NiV or HeV glycoprotein-expressing cells (1×10^5) were mixed with each target cell type (1×10^5) in duplicate wells of a 96-well plate. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. The level of background β-Gal activity in target cell populations alone is indicated as target cells, the level of background β-Gal activity in effector cell populations alone is indicated as no target cells. The β-Gal activity from target cells mixed with HeLa partner cells infected with only T7 RNA polymerase-encoding vaccinia virus and no vaccinia recombinants encoding NiV or HeV glycoproteins is indicated as: none. A. Species tropism of NiV-mediated cell fusion. B. NiV-mediated cell fusion with human T cell and neuroblastoma cell lines as compared to HeV-mediated fusion. This experiment was done twice.

A


no effector cells
none
NiV F
NiV G
NiV F + NiV G
60
45
30
15
0

B


no effector Cells
none
NiV F + NiV G
HeV F + HeV G
100
75
50
25
0
400
300
200
100
0

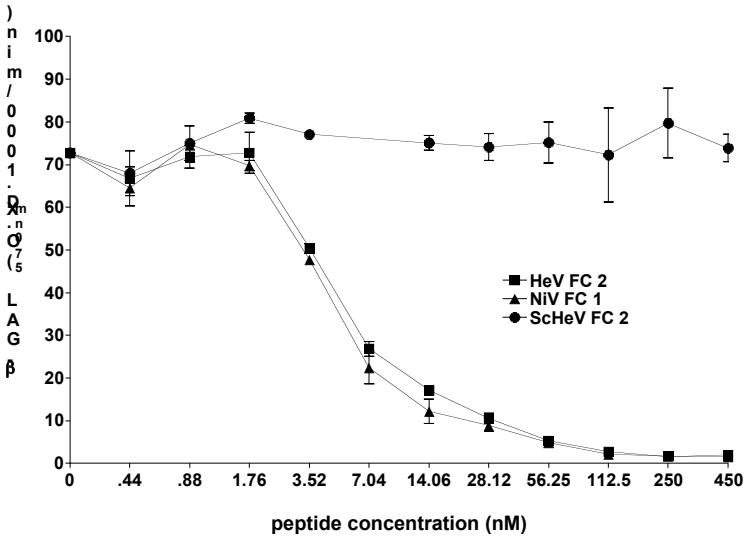
were able to mediate fusion with the same target cell populations used by HeV and for both NiV and HeV, BSC-1, U373, BHK 21 and cat embryo supported the highest level of fusion. Together, these findings suggest that HeV and NiV may use the same receptor on the cell surface of the target cells. Earlier data had already suggested that HeV may be using a cell surface protein as its receptor for fusion and viral entry (124). The broad species tropism demonstrated by HeV and NiV in the cell-fusion assay is a unique biological property that is not common to other paramyxoviruses. Moreover, the large number of species that contain receptor-positive cells may play an important role in the cross species transmission of these viruses from animals to humans. Like previous studies with HeV F and G, NiV F and G were unable to mediate fusion with the pig kidney cell line used in this study. As previously discussed, more pig cell lines need to be tested to further support the notion that our *in vitro* host cell tropism results correlate with natural infections.

Specificity of HeV and NiV-mediated fusion activity. There have been major recent advances in the understanding of the structural requirements and potential mechanisms involved in the fusion of the membrane of enveloped viruses with their host cell membrane (reviewed in: (62, 112, 128, 129)). Current evidence, from a number of groups, supports a model indicating that the formation of a trimer-of-hairpins structure, whose oligomeric coiled-coil formation is mediated by the 2 α-helical heptad repeat domains of the fusion protein, is coupled to membrane fusion. Peptides derived from either of the α-helical heptad repeat regions of enveloped viral fusion proteins have previously been shown to be potent inhibitors of the fusion process for a number of

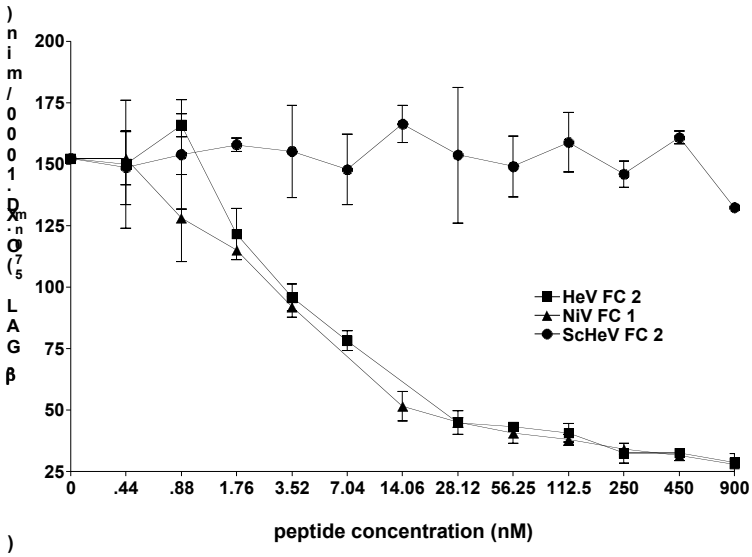
viruses, including several paramyxoviruses, when present during the fusion process (67-70, 72, 122, 130). Both HeV and NiV have two putative heptad repeat domains in F, one proximal to the fusion peptide of F1 (N-terminus), and the other very close to the predicted transmembrane domain (C-terminus). Helical wheel analysis of HeV F revealed a high degree of sequence homology of important functional residues of the heptad repeats of SV5 F, and C-terminal HeV synthetic peptides inhibited HeV-mediated fusion (124). To determine if these domains played an important role in NiV-mediated fusion, a 42 amino acid peptide analogous to the NiV F C-terminus heptad repeat was synthesized (NiV FC1) and tested for its ability to interfere with NiV-mediated fusion. Since there were three amino acid differences within the C-terminus heptad repeat of HeV and NiV, a second peptide corresponding to the HeV F C-terminus heptad repeat was also synthesized (HeV FC2). A scrambled version of HeV FC2 was synthesized and used as a negative control. Shown in **Figure 19A** and **19B** are the results obtained in the presence of these peptides for both HeV and NiV-mediated fusion. HeV FC2 and NiV FC1 could inhibit both HeV- and NiV-mediated fusion in a dose-dependent manner and was completely inhibitory in the nM range, with IC_{50} values between 5.2 and 5.8 nM, respectively. ScHeV FC2 had no inhibitory effect on HeV- or NiV-mediated fusion. These data suggest that HeV and NiV have a similar mechanism of virion-cell membrane fusion, and that this mechanism is likely to be comparable to that proposed for other viral fusion systems, where a trimer of hairpins has been hypothesized to form. There were no significant differences in the ability of HeV FC2 and NiV FC1 to neutralize either HeV- or NiV-mediated fusion. The conservative Y450F or K479R amino acid substitutions of NiV F did not affect the ability of NiV FC1 to inhibit HeV-mediated fusion or the ability

Figure 19. Specificity of NiV and HeV-mediated fusion. Effector cells were prepared as described in the legend of Figure 18. Human U373 cells were infected with the *E.coli* *LacZ*-encoding reporter vaccinia virus vCB21R (target cells). Peptides or rabbit anti-IrNiV serum were diluted and added to the glycoprotein-expressing cells (1×10^5) in a 96-well plate, U373 cells were then added (1×10^5). Each peptide and sera concentration were performed in duplicate in 96-well plate format. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. A. Inhibition of HeV-mediated fusion by C-terminal synthetic F peptides. B. Inhibition of NiV-mediated fusion by C-terminal synthetic F peptides. C. Inhibition of NiV-mediated fusion by anti-IrNiV antiserum. This experiment was done twice.

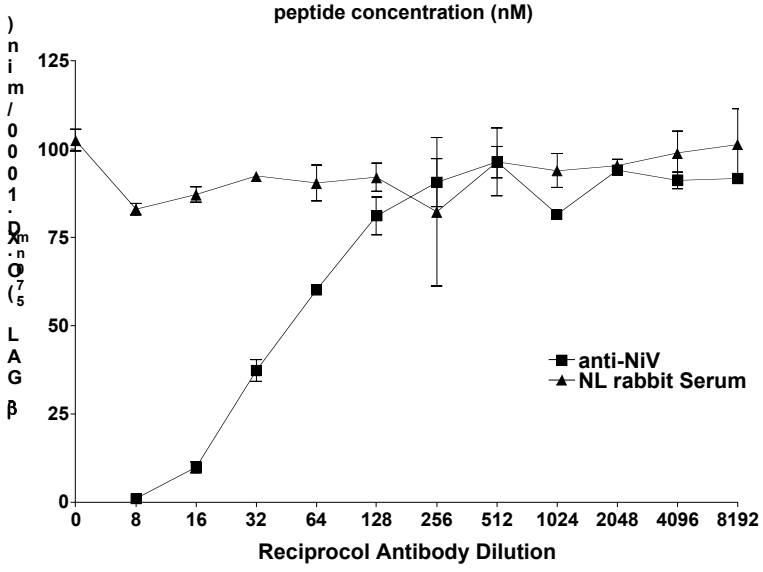
A


HeV FC 2
NiV FC 1
ScHeV FC 2
peptide concentration (nM)

B


HeV FC 2
NiV FC 1
ScHeV FC 2
peptide concentration (nM)

C


anti-NiV
NL rabbit Serum
Reciprocol Antibody Dilution

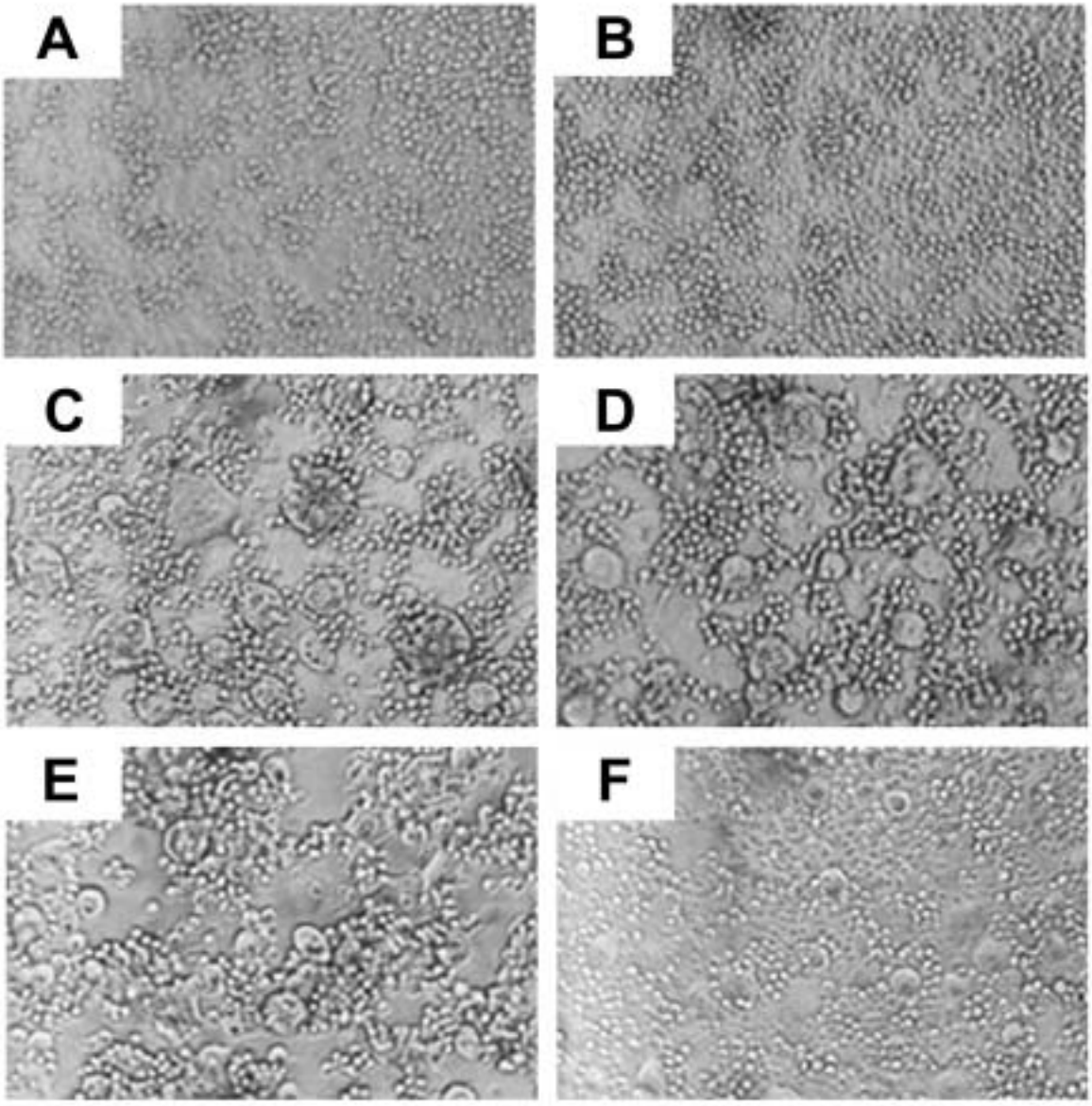
of HeV FC2 to inhibit NiV-mediated fusion. These results are further supported by helical wheel analysis that revealed that none of these amino acids fall in the proposed functional points of the putative C-terminal α-helix of HeV and NiV F thought to be involved in protein-protein interactions leading to the formation of the trimer-of-hairpin fusogenic conformation. NiV FC1, HeV FC2, and ScHeV FC2 had no affect on cell-fusion mediated by the envelope glycoproteins of MeV or CDV (data not shown), further demonstrating the specificity of this recombinant HeV and NiV-mediated membrane fusion system.

To evaluate further the specificity of NiV-mediated fusion, a rabbit anti-IrNiV antiserum and a normal rabbit serum were compared for their abilities to inhibit NiV-mediated cell-fusion. Both sera were diluted serially and added to envelope glycoprotein-expressing effector cell populations just prior to the addition of target cells. The normal rabbit serum slightly lowered NiV-mediated fusion, but by no more than ~15% at the highest sera concentration; conversely, the NiV-specific antiserum could block cell-fusion by >90% at a1: 8 dilution and there was approximately 50% inhibition at a 1:50 dilution (**Figure 19C**). The NiV-specific antiserum was also able to block HeV-mediated cell fusion but to a lesser extent (data not shown). This is probably due to the polyclonal nature of the anti-IrNiV antiserum and the level of antigenic relatedness between HeV and NiV.

Heterologous fusion activity of the HeV and NiV F and G glycoproteins. Since the cellular tropism and fusion requirements of HeV and NiV appeared to be very similar to one another, yet distinct from other paramyxoviruses, we explored whether the envelope

glycoproteins from these henipaviruses could function in the context of heterologous combinations of the fusion and attachment envelope glycoproteins. For most paramyxoviruses, including HeV and NiV, efficient membrane fusion requires the presence of both the fusion and attachment envelope glycoproteins, although there is considerable evidence that fusion mediated by F alone can readily be measured with the F glycoprotein of SV5 (reviewed in (62)). Methods that facilitate close membrane-to-membrane contact can also enhance membrane fusion in the absence of the homotypic attachment protein, an F alone fusion system (131). Among the paramyxoviruses, members of the genus *Morbilliviruses* are the most closely related to HeV and NiV (32). Previously, two morbilliviruses were examined, MeV and CDV, and heterologous function with different combinations of the MeV and CDV envelope glycoproteins was demonstrated (45). Here, in a similar fashion, we examined the ability of the HeV and NiV envelope glycoproteins to function in heterologous combinations in a syncytia formation assay. Shown in **Figure 20** are the syncytia formation results with U373 target cells mixed with HeLa effector cells expressing several HeV and NiV heterologous envelope glycoproteins combinations. The human U373 cells were chosen because of the high level of cell-fusion observed with both HeV and NiV, presumably due to expression of high levels of virus receptor. Homotypic glycoprotein combinations are shown in **Figure 20C and 20D** with HeV and NiV respectively, and efficient cell-fusion with the U373 target cell was evident for both. No syncytia were observed with HeLa effector cells expressing only the F glycoprotein of either HeV or NiV (**Figure 20A and 20B** respectively). Effector cells expressing heterotypic mixes of the F and G glycoproteins of

Figure 20. Syncytia formation mediated by homotypic and heterotypic NiV and HeV envelope combinations. HeLa cells were infected with vaccinia virus recombinants encoding the HeV F (Panel A), NiV F (Panel B), HeV F/HeV G (Panel C), NiV F/NiV G (Panel D), HeV F/NiV G (Panel E), or NiV F/HeV G (Panel F) (effector cells). Partner U373 cells were detached using EDTA and washed 3 times with PBS. The effector cells ($1 \text{ X } 10^5$) were mixed with the U373 partner cells ($1 \text{ X } 10^5$) in duplicate wells of a 96-well plate and incubated at 37°C. After 18 hours, photographs were taken at 400X magnification. This experiment was done twice.

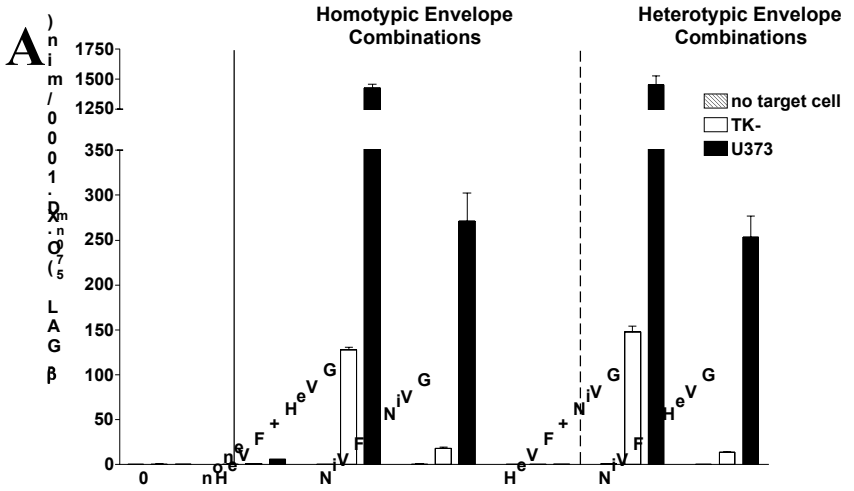


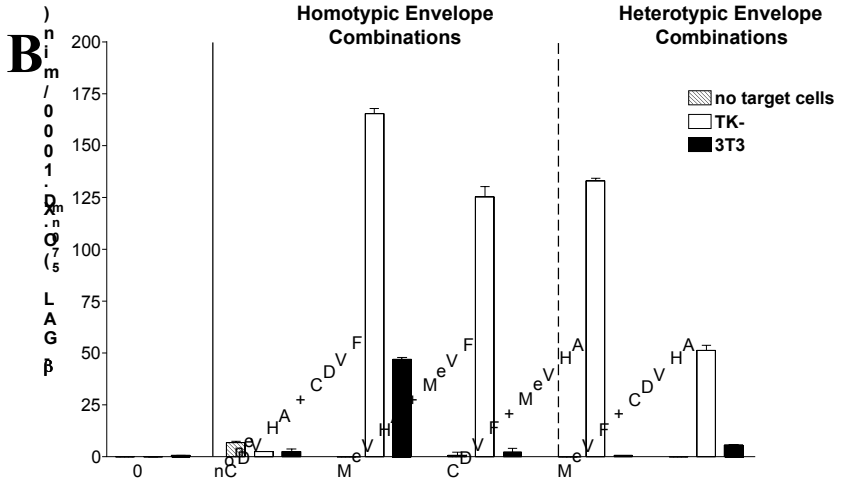
HeV and NiV were clearly capable of mediating cell-fusion with the U373 target cells (**Figure 20E and 20F**). It was also evident that HeV-mediated cell-fusion resulted in

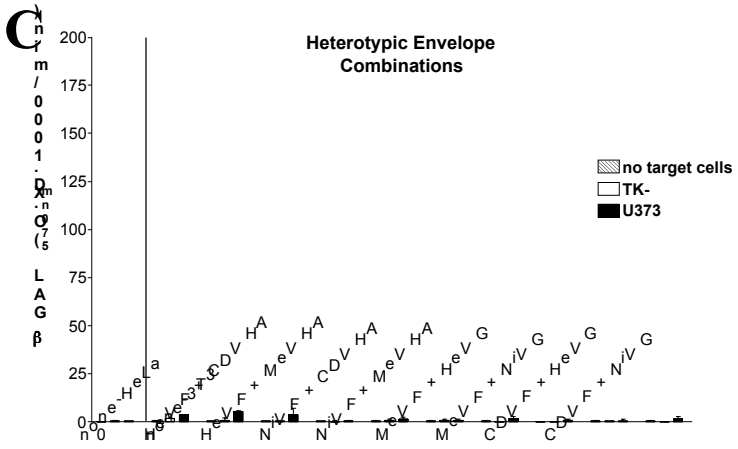
somewhat larger and more numerous syncytia in comparison to those resulting from NiV-mediated cell-fusion.

In light of the results that indicated functional compatibility of the HeV and NiV glycoproteins, as well as the enhanced cell-fusion mediated by HeV F as compared to NiV F, we sought to examine these cell-fusion processes in a quantitative manner to define any subtle differences between homologous and heterologous envelope combinations. Shown in **Figure 21** are quantitative cell-fusion results mediated by effector cell populations expressing various combinations of the HeV, NiV, MeV, and CDV envelope glycoproteins. The NiV and HeV envelope glycoproteins could efficiently mediate fusion in heterologous envelope combinations with themselves (**Figure 21A**). Further, the fusion signals observed with either heterologous combination correlated quite well to the fusion level seen with the homologous combination and appeared to be dependent on the F glycoprotein employed. These fusion results were also in agreement with the syncytia formation results shown in **Figure 20**. Because HeV and NiV were so efficient in supporting heterotypic envelope glycoprotein-mediated fusion, we wanted to examine whether they could also support a heterotypic fusion reaction with glycoproteins derived from other related viruses. In parallel, we re-assessed our heterotypic fusion results using the fusion and attachment glycoproteins from the morbilliviruses MeV and CDV (**Figure 21B**). As expected, heterotypic combinations of MeV and CDV envelope glycoproteins were capable of mediating fusion, although less efficiently than the homologous envelope combinations. Here, MeV is permissive for

Figure 21. Quantitation of cell fusion mediated by homotypic and heterotypic NiV and HeV envelope combinations. HeLa or 3T3 cells were infected with vaccinia virus recombinants encoding the following envelope glycoproteins, HeV F, HeV G, NiV F, NiV G, MeV F, MeV HA, CDV HA, or CDV F, in various combinations, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). HeLa cells were used as effector cells for expression of HeV and NiV G envelope combinations; 3T3 cells were used as effector cells for expression of MeV and CDV HA envelope combinations. TK^-, U373, and 3T3 target cells were infected with the *E.coli LacZ*-encoding reporter vaccinia virus vCB21R (target cells). The glycoprotein-expressing cells (1×10^5) were mixed with each target cell type (1×10^5) in duplicate wells of a 96-well plate. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. The level of background β-Gal activity in target cell populations alone is indicated as target cells, the level of background β-Gal activity in effector cell populations alone is indicated as no target cells. The β-Gal activity from target cells mixed with effector cells infected with only T7 RNA polymerase-encoding vaccinia virus and no vaccinia recombinants encoding glycoproteins is indicated as: none. A. HeV and NiV envelope combinations. B. MeV and CDV envelope combinations. C. *Henipavirus* and *Morbillivirus* envelope combinations. All envelope glycoprotein combinations were within the same experiment. This experiment was done twice.

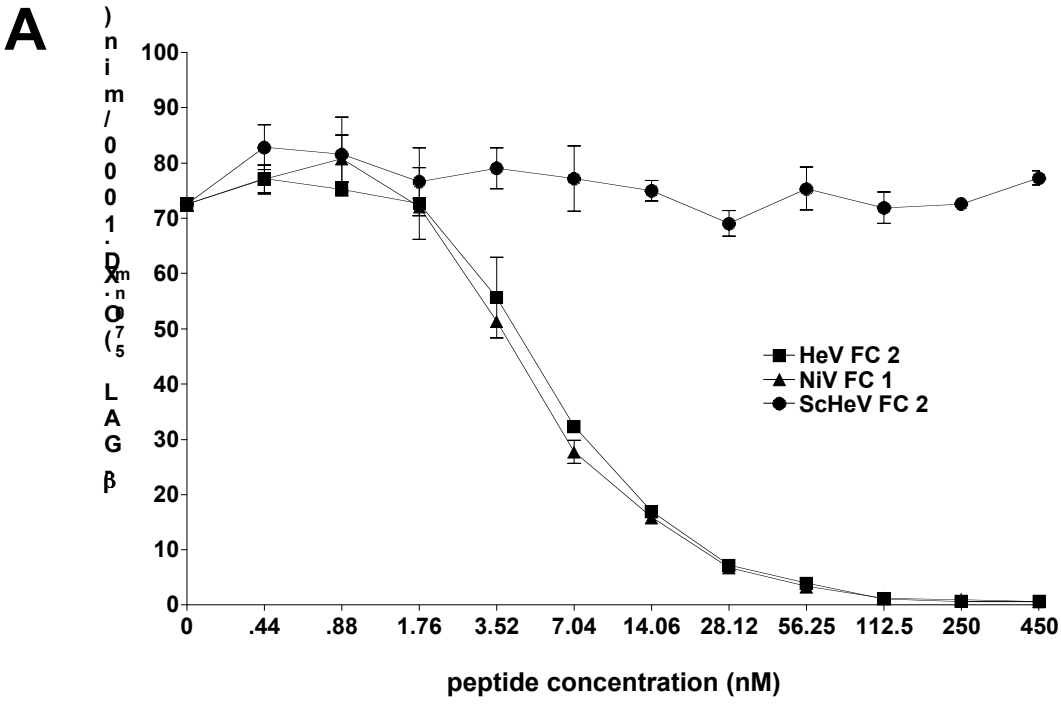

A
Homotypic Envelope Combinations
Heterotypic Envelope Combinations
no target cell
TK-
U373
1750
1500
1250
350
300
250
200
150
100
50
0


B
Homotypic Envelope Combinations
Heterotypic Envelope Combinations
no target cells
TK-
3T3
200
175
150
125
100
75
50
25
0


C
Heterotypic Envelope Combinations
no target cells
TK-
U373
200
175
150
125
100
75
50
25
0

fusion with the human TK- cells while the murine 3T3 cells are not, owing to the absence of a functional MeV receptor. Whereas, the CDV can mediate fusion with both target cell types. We then tested whether coexpression of NiV and HeV F or G with morbillivirus F or H from MeV and CDV could result in any heterotypic fusion activity; however, no fusion was detectible with any glycoprotein combination (**Figure 21C**). The fact that the heterotypic results with these related morbilliviruses is much less efficient than that observed with HeV and NiV is consistent with the notion that HeV and NiV are likely using the same cell surface receptor while MeV and CDV, although closely related viruses, are not. The fusion specificity observed with the HeV and NiV heterotypic functional activity was verified using HeV FC2 and NiV FC1 as was done for homologous envelope combinations and results are shown in **Figure 22**. HeV F/NiV G-mediated fusion was completely inhibited by HeV FC2 and NiV FC1 and the dose-dependent curve closely resembled that seen with HeV F/HeV G. NiV F/HeV G-mediated fusion was also completely inhibited by HeV FC2 and NiV FC1 and the dose-dependent curve resembled that seen with NiV F/NiV G. The IC_{50} calculations for inhibition of all HeV/NiV envelope-mediated fusion by HeV FC2 and NiV FC1 are summarized in Table 2.

Figure 22. Specificity of heterotypic envelope function. Effector cells were prepared as described in the legend of Figure 18. Human U373 cells were infected with the *E.coli LacZ*-encoding reporter vaccinia virus vCB21R (target cells). C-terminus heptad derived peptides were diluted and added to the glycoprotein-expressing cells (1×10^5) in a 96-well plate, U373 cells were then added (1×10^5). ScHeV FC2, a scrambled version of the HeV FC2 peptide, was used as a negative control. Each peptide and sera concentration was performed in duplicate in 96-well plate format. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. A. Inhibition of NiV G/HeV F-mediated fusion by C-terminal synthetic F peptides. B. Inhibition of NiV F/HeV G-mediated fusion by C-terminal synthetic F peptides. This experiment was done twice.


A
100
90
80
70
60
50
40
30
20
10
0
0
.44
.88
1.76
3.52
7.04
14.06
28.12
56.25
112.5
250
450
peptide concentration (nM)
HeV FC 2
NiV FC 1
ScHeV FC 2

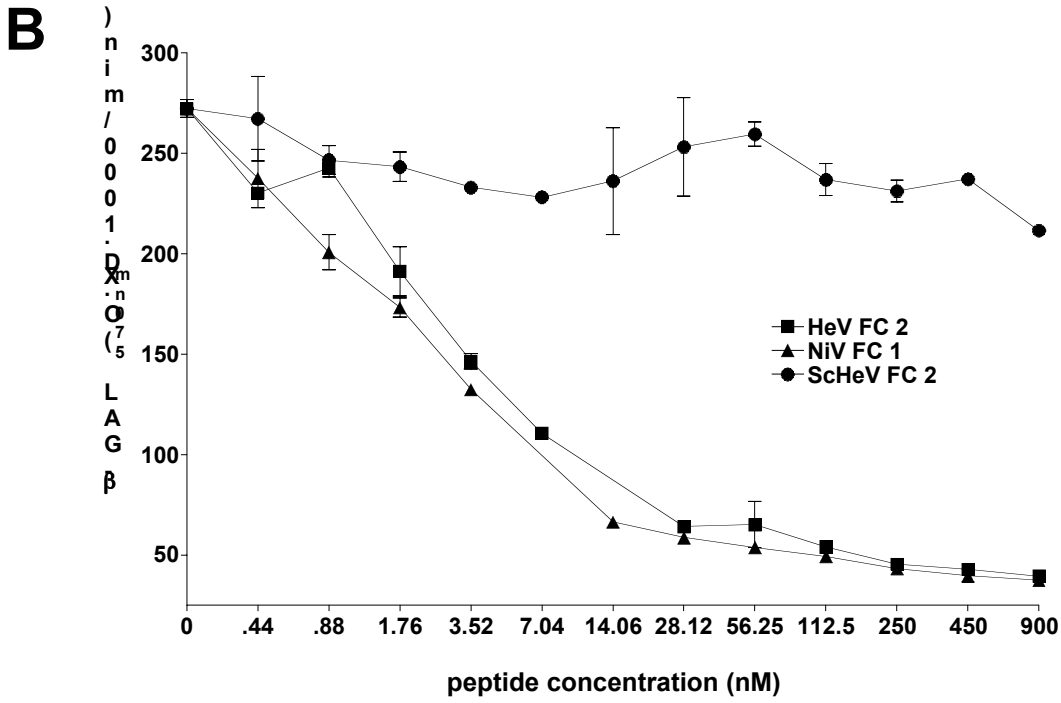

B
300
250
200
150
100
50
0
.44
.88
1.76
3.52
7.04
14.06
28.12
56.25
112.5
250
450
900
peptide concentration (nM)
HeV FC 2
NiV FC 1
ScHeV FC 2

Table 2. IC_{50} of peptides derived from the fusion glycoproteins of HeV and NiV

Envelope Combination	**HeV FC 2 IC_{50}**[a]	**NiV FC 1 IC_{50}**[b]
HeV F + HeV G	5.8 nM	5.2 nM
HeV F + NiV G	6.5 nM	5.9 nM
NiV F + NiV G	5.3 nM	5.8 nM
NiV F + HeV G	2.5 nM	2.9 nM

a HeV FC 2 is specific to the C-terminal heptad repeat of HeV F
b NiV FC 1 is specific to the C-terminal heptad repeat of NiV F

DISCUSSION

The results presented herein have established the requirements for NiV-mediated fusion and have defined some functional similarities and differences between the NiV and HeV envelope glycoproteins. These two viruses emerged in two geographically isolated countries, five years apart. However, they appear to have the same reservoir in nature; i.e. certain Australian fruit bat species, commonly known as flying foxes (19-21, 132-134). As a group, these animals have a large range, which encompasses much of the South Pacific, spreading as far west as the eastern coast of Africa. It is also of interest that these two viruses independently spread from different animal species to cause fatal disease episodes in humans. Thus, in light of the evidence suggesting the existence of additional and distinct Hendra-like viruses in various pteropid bat species, these observations suggest additional emerging viruses may yet appear as other animal species serve as amplifying hosts. It is of importance to understand the mechanisms that underlie the transmission of such new infectious agents in nature to both animals and, ultimately, to humans. HeV caused a fatal respiratory disease in 14 horses, and although only two human cases were diagnosed at that time, the unknown disease in such a large number of horses lead to a further investigation and the eventual discovery of the virus. Nipah, however, did not cause fatal disease in pigs, but the large outbreak of human cases necessitated the investigation into the causative agent, which was then traced to infected pigs in close contact with humans. The diseases seen in animals and humans is different between these two viruses, but their genetic make-up and some similar biological properties show their relatedness. Here we report studies detailing several functional differences and commonalities between NiV and HeV envelope glycoproteins.

The NiV envelope glycoproteins were cloned into vaccinia shuttle vectors and recombinant vaccinia viruses were made. Envelope expression was verified through metabolic labeling and immunoprecipitation using anti-IrNiV antiserum. The molecular weight of the recombinant expressed NiV G was comparable to that seen with HeV G migrating ~75 kDa and had a slower mobility than its predicted molecular weight of ~67 kDa. The molecular weight of the recombinant expressed NiV F_0 precursor was ~61 kDa, the processed F_1 subunit was ~49 kDa. and the processed F_2 subunit was ~19 KDa. The recombinant expressed NiV F glycoprotein appeared to be processed to a greater extent than the HeV F glycoprotein, in agreement with observations made with infectious virus (32). Based on the similarity in molecular weights in comparison to other members of the *Paramyxoviridae,* the F and G glycoproteins of NiV are undoubtedly N-glycosylated at one or more sites.

Species cell-fusion tropism for NiV was examined and compared to HeV and both were found to use the same receptor recognition pattern among the cell lines we examined, and those that supported the highest level of HeV-mediated fusion also supported the highest level of NiV-mediated fusion. These data suggest that HeV and NiV most likely use the same receptor for virus entry into receptive host cells. Our previous work also suggested that HeV may be using a cell surface protein as its receptor (124). The human U373 cell line supported the highest level of membrane fusion for both NiV and HeV. This observation is of interest since this is a human cell line of neuronal origin, and it suggests that related cell types could be important targets in natural human infections. Indeed, the significant difference between NiV-mediated fusion with U373 cells and other target cells is of particular interest due to the enhanced neuropathology seen in NiV-infected

individuals. Although only one HeV- infected individual died with obvious neuropathology, the number of HeV infected humans was quite small compared to the outbreak of NiV and neurotropism of HeV could also be an important feature of HeV infection in humans.

Another observation of interest from our studies was that HeV-mediated fusion was consistently more potent than NiV-mediated fusion. Since we believe that HeV and NiV may share the same receptor, the difference in the potency of fusion may be attributable to either structural/functional differences in the F and G envelope glycoproteins or, alternatively, to the ways that the two proteins engage one another. The recombinant vaccinia virus-expressed NiV F glycoprotein appeared to be processed to a greater extent than the HeV F glycoprotein, in agreement with observations made with infectious NiV (32). Indeed, although NiV and HeV are quite closely related viruses on a genetic basis, the cleavage recognition site of the HeV F precursor polypeptide contains a lysine (K) residue in the P1 position whereas the NiV F precursor contains an arginine (R) in that position, which is similar to all other fusion glycoproteins among other *Paramyxoviridae* members and across several virus families including the *Orthomyxoviridae*, *Flaviviridae*, *Togaviridae*, and *Retroviridae*, (50). Although this is a conservative amino acid substitution, it may be important for proteolytic cleavage and activation of F. Mutagenesis studies are underway to determine what role this cleavage site distinction plays in F_0 processing and the subsequent fusion rates seen between both HeV and NiV F. Indeed, the fusion rates demonstrated here in the heterologous mixing experiments support the notion that the F envelope glycoprotein is the rate-limiting component in the mechanism affecting the fusion activity.

The basis of the observed fusogenic differences between HeV and NiV is not clear. Even though processing of F_0 is necessary for its activation and there are apparent functional differences between HeV and NiV F, it is also possible that there are functional differences in the efficiency of each F glycoprotein to mediate fusion. Alternately, there may also be differences in how the HeV and NiV F and G interact or engage with one another to mediate the fusion event. There are two amino acid differences in the predicted fusion peptide sequence but these differences do not seem likely candidates to account for the fusogenic differences since they are conservative changes; i.e. isoleucine (Ile) for valine (Val) at position 114 and Val for Ile at position 118 (82). We examined the fusion specificities for homologous and heterologous envelope combinations by using peptides derived from the C-terminus α-heptad repeat from either HeV or NiV F. However, we observed no significant differences in the IC_{50} values (**Table 1**) for either peptide in either the homologous or heterologous envelope combinations. These data would suggest that both the trimer-of-hairpins formation and the conformational structure of each F glycoprotein involved in the fusion process is likely conserved between HeV and NiV. As with other viral glycoprotein-mediated fusion models, it is not known if the 6-helix bundle formation immediately precedes fusion, occurs concurrently with fusion, or occurs after fusion. Nevertheless, the data presented here regarding the inhibition of the fusion processes of HeV and NiV offer an attractive avenue for the development of novel therapeutics. This approach has met with promising success with HIV-1 (135), and the peptides derived from the C-terminus heptad repeat of HeV and NiV F are capable of the inhibition of infectious HeV and NiV entry (Eaton et al., unpublished results).

Another possibility that may account for differences in the fusion efficiency between HeV and NiV would be structural differences in the F and G envelope glycoproteins, or co-translational modifications of, these glycoproteins that affect their function. Such structural differences or modifications of HeV F may allow it to interact with either the HeV or NiV G glycoprotein more efficiently than NiV F and account for the increased rate of fusion. Although there are significant differences in the amino acid sequences of HeV and NiV G glycoproteins, the critical residues necessary to contact and/or induce conformational changes in either HeV or NiV F appear to be present between the two G proteins based on the efficiency of heterotypic fusion activity observed here. The delineation of the regions in both F and G that are involved in their interaction in mediating membrane fusion will aid in our understanding of the mechanism of paramyxovirus fusion in general. Preliminary studies in our laboratory have revealed an N-linked glycosylation site deletion mutant of HeV G that is no longer capable of supporting efficient HeV-mediated fusion (Broder and Bossart, unpublished results). This type of co-translational modification may be critical in determining the native structure of G or perhaps play an important role in the interaction between HeV F and G, and/or act to stabilize the proposed fusogenic conformation of HeV F. The HeV and NiV G glycoproteins share only 83% amino acid identity, yet they are identical in the location and number of 7 extracellular, potential N-linked glycosylation sites (32, 82) suggesting that certain sites may be critical for proper folding or function of the glycoprotein.

In summary, we have established a recombinant system to express and characterize the F and G membrane glycoproteins of NiV and HeV. This system has afforded the opportunity to examine these glycoproteins on a functional level in a quantitative manner

and will also serve as a useful tool in future experiments aimed at exploring the interactions between the F and G glycoproteins. We have also demonstrated that efficient NiV-mediated membrane fusion requires both the F and G glycoproteins as was observed for HeV and most all other paramyxoviruses. NiV-mediated fusion has demonstrated a broad species tropism similar to results obtained with HeV. In addition, the results presented here have indicated that HeV-mediated fusion is more potent or efficient than that of NiV. The membrane fusion mechanism shown here by NiV as well as HeV can be specifically inhibited with either antisera or targeted peptides, and this system may prove useful as a surrogate assay for measuring immune-based inhibition of virus infection outside of BSL-4 containment. Taken together, these functional studies have laid the foundation for a variety of approaches, which may be followed for reagent development and for exploring the fusion and attachment glycoprotein functions of these interesting and unique emerging paramyxoviruses.

Chapter 5

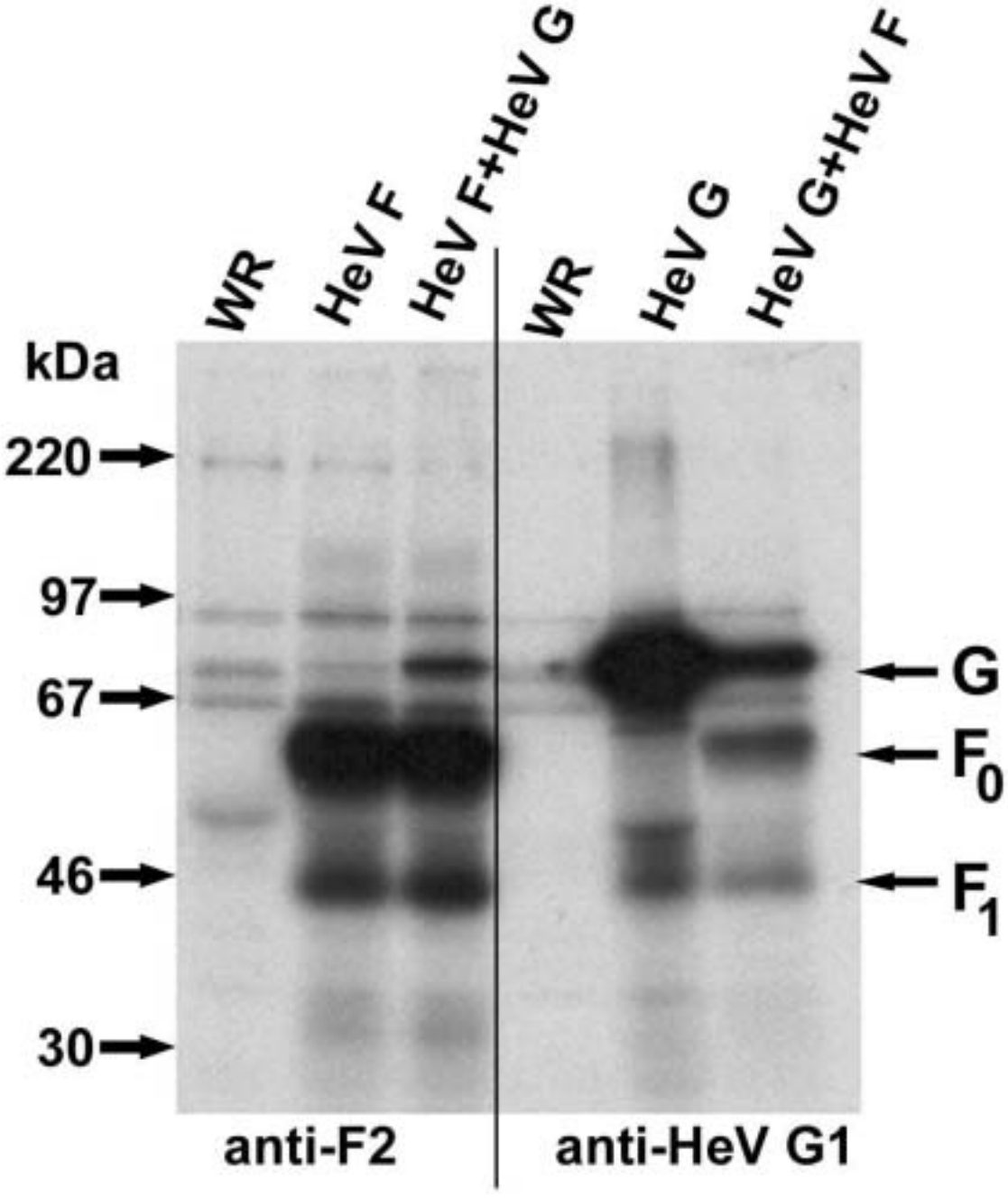
Co-Immunoprecipitation of the Fusion and Attachment Envelope Glycoproteins of Hendra Virus and Nipah Virus

RESULTS

Co-Immunoprecipitation of Hendra virus F and G glycoproteins. The mechanism that underlies the functions of the fusion and attachment glycoproteins of the paramyxoviruses in the membrane fusion process has remained elusive for a number of years. A long-standing hypothesis has been that there is a physical association between these glycoproteins while present in the membrane of the virus or on the surface of an infected cell. Upon engagement of the attachment glycoprotein to an appropriate cellular receptor, there is some form of activation process, which is relayed to the F glycoprotein to trigger its fusogenic activity (61, 62). An obvious approach to investigate this possibility is through a co-precipitation type of experiment where isolation of the viral fusion and attachment envelope glycoproteins is specific for only one glycoprotein and detection is then directed to the other or to both. The strength of the interaction between the fusion and attachment glycoproteins is quite variable among the different paramyxoviruses that have been examined to date, and often the application of certain chemical cross-linking agents has been applied to this kind of analysis (58-60). For the most part, this approach has met with only marginal success with several paramyxovirus species. In previous work, we have determined that the HeV envelope glycoproteins were more efficient at mediating membrane fusion than the NiV envelope glycoproteins. Moreover, we reported that the HeV and NiV glycoproteins could complement each other in a heterotypic fashion, and that HeV F-containing envelope glycoprotein combinations

consistently mediated the most potent levels of membrane fusion (136). Because of the unique feature of these two new paramyxoviruses (HeV and NiV) of highly efficient heterotypic membrane fusion in comparison to all other paramyxoviruses that have been examined to date (45, 53), we hypothesized that they may possess the property of a more potent protein-protein interaction between their fusion and attachment glycoproteins. We sought to examine their F and G glycoproteins to determine whether they are better able to demonstrate the predicted physical association between a paramyxovirus fusion and attachment envelope glycoprotein. We speculated that the higher levels of membrane fusion activity mediated by HeV F could be due to its ability to strongly interact with its matched G glycoprotein. Further, we hypothesized that NiV-mediated fusion is less vigorous in comparison due to a weaker interaction between its F and G glycoproteins. Since we had determined that HeV F was able to function equally well with either HeV G or NiV G, we predicted that HeV F would interact to an equivalent extent with either. In contrast, NiV F would only have weaker interactions with either G glycoprotein. In previous studies we developed specific antisera that were capable of immunoprecipitating either the HeV F or G envelope glycoprotein (124) and also, that certain cell lines, such as HeLa, do not possess a functional receptor, as they were not capable of serving as a fusion permissive target cell. We utilized these antisera to initiate an examination of the biochemical interaction of HeV F and G. The results from one of these initial co-immunoprecipitation experiments is shown in **Figure 23,** which clearly demonstrates that HeV F and G are able to physically associate in the absence of receptor and in a non-fusogenic state. HeLa cells were infected with wildtype vaccinia virus strain, WR (control) or with vaccinia virus recombinants encoding

Figure 23. **Co- immunoprecipitation of the HeV F and G glycoproteins.** HeLa cells were infected with HeV F- or G-encoding recombinant vaccinia viruses and incubated 16 h at 37°C. Beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine for immunoprecipitation. Lysates were prepared in buffer containing Triton X-100 and clarified by centrifugation. Immunoprecipitation was performed with rabbit anti-HeV F_2 or rabbit anti-HeV G1 antisera followed by Protein G-Sepharose. The metabolically labeled proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by fluorography. Lane 1: WR-control vaccinia virus; lane 2: recombinant expressed HeV F; lane 3: recombinant expressed HeV F + HeV G; lane 4: WR- control vaccinia virus; lane 5: recombinant expressed HeV G; lane 6: recombinant expressed HeV F + HeV G. Lanes 1-3 were immunoprecipitated with a serum specific for HeV F; lanes 4-6 were immunoprecipitated with a serum specific for HeV G (see Materials and Methods). This experiment was done twice.


WR
HeV F
HeV F+HeV G
WR
HeV G
HeV G+HeV F
kDa
220
97
67
46
30
G
F0
F1
anti-F2
anti-HeV G1

either HeV F alone, HeV G alone or HeV F and G. For all combinations, lysates were prepared in buffer containing 1% Triton X-100 and clarified by centrifugation. Immunnoprecipitations in the left panel were done with anti-F_2 antiserum, whereas the right panel was immunoprecipitated with anti-HeV G1 antiserum. For both panels the specific antisera are capable of immunoprecipitating either HeV F or HeV G alone. The molecular weights of each protein are 61 and 49 kDa for F_0 and F_1, respectively, and 75-80 kDa for HeV G. When HeV F and G were co-expressed and immunoprecipitated with anti-F_2, a band that migrated very close to the molecular weight of G intensified, as seen in lanes 2 and 3 of the right panel. When HeV F and G were co-expressed and immunoprecipitated with anti-HeV G1 antiserum two additional bands appeared which had the same molecular weight as F_0 and F_1. Previous studies have demonstrated that over-expression of the F glycoprotein of paramyxoviruses is capable of down-regulating the expression of the homologous attachment glycoprotein (137). This down-regulation may explain why G glycoprotein expression, in cells co-infected with recombinant vaccinia viruses that express both the F and G glycoproteins, is much reduced as compared to cells infected with a recombinant vaccinia virus that expresses the G glycoprotein alone. Together our data demonstrated that the HeV F and G biochemical interaction is readily detectable. We also observed that their interaction was fairly strong because it remained intact throughout the washing of the immunoprecipitates which included one ionic wash with 0.1% DOC / 0.1%SDS in addition to the 1% Triton X-100 in the wash buffer.

Expression of radiolabeled epitope-tagged HeV and NiV envelope glycoproteins. Unfortunately, the anti-NiV G1 antiserum that we previously made was unable to immunoprecipitate NiV G and could only be used in Western blot analysis. Moreover, although the amino acid sequence of the F_2 peptide used to make the anti-HeV F_2 antiserum was identical in HeV and NiV F, this antiserum was unable to immunoprecipitate NiV F. This confounded our ability to replicate the co-immunoprecipitation experiment shown in **Figure 23** using the NiV envelope glycoproteins. Therefore to facilitate the continuation of these studies that were aimed at dissecting the interactions between the F and G glycoproteins of each virus, we designed several epitope-tagged versions of each molecule. We chose to employ two widely used epitope-peptide sequences derived from the human c-myc protein, N-terminus-EQKLISEEDL-C-terminus (myc) and the influenza hemagglutinin sequence, N-terminus-YPYDVPDYA-C-terminus (HA). The HeV and NiV G envelope glycoproteins were tagged with the myc epitope at their C-terminus. The ectodomains, or the molecules' C-terminus, were chosen in light of the observations by Plemper et al. (138), that an 8 amino acid FLAG tag addition to the cytoplasmic tail unexpectedly reduced the interaction between MeV F and H and resulted in corresponding increase their membrane fusogenic activity. The HeV and NiV F envelope glycoproteins were tagged originally with HA at their N-terminus, also the ectodomain of the molecule. Although these HA epitope-tagged proteins were expressed at wild-type levels, they unexpectedly were non-functional (data not shown). Therefore the HA tag was moved to the C-terminus extending the length of their cytoplasmic tails. All epitope tags were added as translational fusions by PCR and then each product was subcloned into the vaccinia

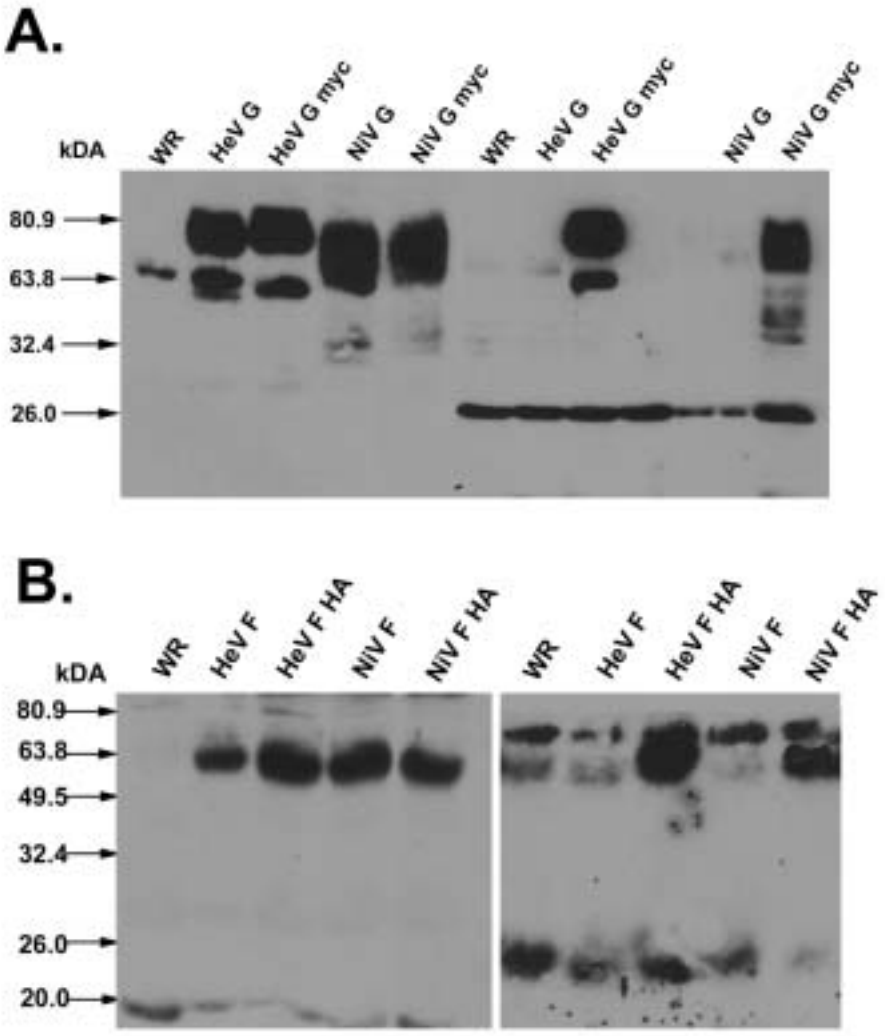
shuttle vector pMC02 (90) and recombinant vaccinia viruses were generated using standard procedures (see Materials and Methods). The epitope-tagged envelope glycoproteins were produced in culture by infection of HeLa cells with the appropriate recombinant vaccinia virus. Beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine, and then chased for 2 hours in complete medium. Shown in **Figure 24**, **Panel A** is HeV G-myc immunoprecipitated with virus-specific antiserum, peptide-specific antiserum, or myc epitope-specific antibodies (Mab). All three antibody precipitations were able to precipitate HeV G-myc that possessed an apparent molecular weight of ~75-80 kDa, essentially identical to that seen of HeV G. **Figure 24, Panel B** is NiV G-myc immunoprecipitated with virus-specific antiserum, peptide-specific antiserum, or myc Mab. The virus-specific antiserum and myc Mab were able to precipitate NiV G-myc, that possessed an apparent molecular weight of ~70-75 kDa, essentially identical to that seen of NiV G. There is a non-specific protein (band), which appears in the lane precipitated with anti-NiV G1, detected in the WR vaccinia virus control lysates. Also apparent is that HeV G-myc migrates slightly more slowly then NiV G-myc, which was also observed in a comparison of the wild-type HeV G and NiV G, where HeV G migrates slightly more slowly. The anti-myc epitope antibody was able to precipitate as much HeV G-myc or NiV G-myc as the virus-specific antisera in both cases. Recombinant expressed HeV F-HA and NiV F-HA radioimmunoprecipitations are shown in **Figure 24, Panel C**. Lanes 1-3 are WR controls, lanes 4-6 are HeV F-HA, and lanes 7-10 are NiV F-HA. Each group was immunoprecipitated with virus-specific antisera, peptide-specific antisera, or HA Mab. For both HeV F-HA and NiV F-HA a band with an apparent molecular weight of

Figure 24. Expression of recombinant HeV and NiV F-HA and G-myc epitope-tagged glycoproteins. Epitope-tagged envelope glycoproteins were generated using PCR and then subcloned into the vaccinia shuttle vector pMC02 (90) and recombinant vaccinia viruses were made (see Materials and Methods). Each F protein contains an HA tag on the cytoplasmic tail of the protein, whereas each G protein contains a myc tag at the C-terminus in the ectodomain of the protein. HeLa cells were infected with F-HA or G-myc encoding viruses and incubated 16 h at 37°C. Beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine. Lysates were prepared in buffer containing Triton X-100 and clarified by centrifugation. Immunoprecipitation was performed with rabbit anti-IrHeV, rabbit anti-IrNiV, rabbit anti-HeV G1, rabbit anti-NiV G1 antiserum, or rabbit anti-F_2 antiserum, or commercial mouse anti-myc or mouse anti-HA monoclonal antibodies followed by Protein G-Sepharose. The metabolically labeled proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by fluorography. Panel A: lanes 1-3: WR-control vaccinia virus; lane 4-6: recombinant expressed HeV G-myc; Panel B: lanes 1-3: WR-control vaccinia virus; lane 4-6: recombinant expressed NiV G-myc; Panel C: lanes 1-3: WR-control vaccinia virus; lane 4-6: recombinant expressed HeV F-HA; lanes 7-9: recombinant expressed NiV F-HA. This experiment was done twice.

~61 kDa was observed with all three antibodies, essentially identical to the molecular weight of wild-type HeV and NiV F_0. For NiV F-HA the virus-specific antiserum also precipitated F_1 with a molecular weight of ~49 kDa. Surprisingly, the NiV F-HA glycoprotein was also immunoprecipitated by the anti-F_2 peptide-specific antiserum, unlike wild-type NiV F. Perhaps the addition of the HA tag to the cytoplasmic tail of NiV F has slightly altered the conformation of the glycoprotein further exposing the F_2 epitope.

Expression of epitope-tagged HeV and NiV envelope glycoproteins detected by Western blot. Although the radioimmunoprecipitations confirmed expression of the epitope-tagged proteins, it was important to be able to detect these proteins specifically by Western blot analysis. As before, the wild-type and epitope-tagged envelope glycoproteins were produced in culture by infection of HeLa cells with the appropriate recombinant vaccinia virus, followed by an overnight incubation in fresh medium. Cell lysates were prepared as described above and 25 μl of each lysate was subjected to 10% SDS-PAGE. Gels were transferred overnight to nitrocellulose and then probed with the appropriate antibody. **Figure 25 Panel A** shows wild-type HeV and NiV G and epitope-tagged HeV and NiV G-myc. Lanes 1-5 were probed using a mixture of HeV G1 and NiV G1 antisera, lanes 6-11 were probed with the myc Mab. Both HeV G-myc and NiV G-myc were noted to migrate slightly more slowly than their wild-type counterpart due to the addition of the 10 amino acid epitope tag. Furthermore, the myc Mab specifically recognizes only the myc tagged G glycoproteins and again illustrated that HeV G-myc migrates slightly slower than NiV G-myc. Detection of the wild-type HeV and NiV F

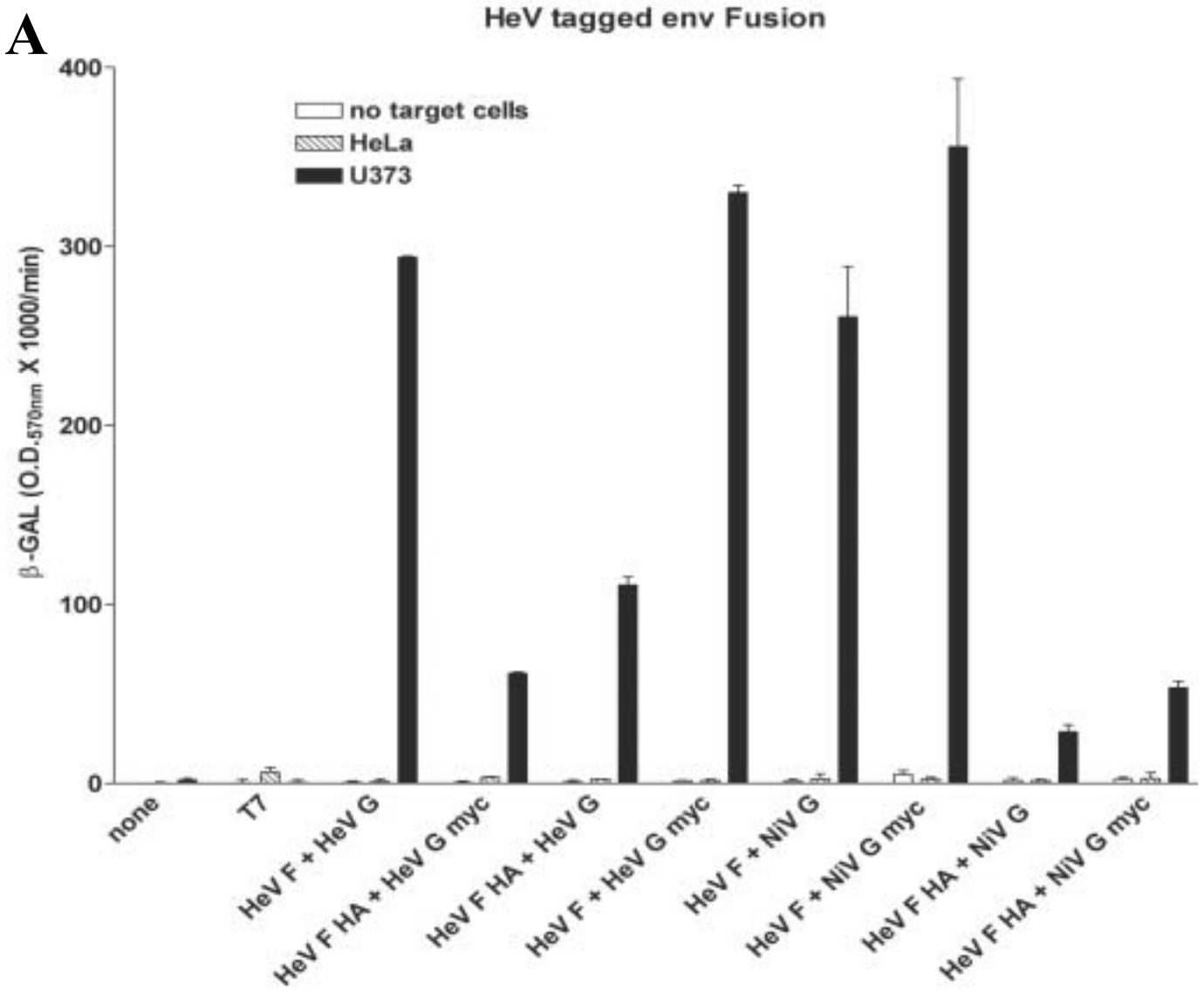
Figure 25. Western blot analysis of recombinant HeV and NiV F-HA and G-myc epitope-tagged glycoproteins. HeLa cells were infected with HeV or NiV G-myc or HeV or NiV F-HA encoding viruses and incubated 2 h at 37°C. Infected cells were washed twice, 1 ml of fresh medium was added and infections were incubated overnight. Lysates were prepared in buffer containing Triton X-100 and clarified by centrifugation. Western Blot analysis was performed using rabbit antisera raised against a synthetic F_2 peptide, a synthetic HeV G1 peptide, or a synthetic NiV G1 peptide, or a mouse monoclonal α-myc antibody. The HA-tagged F proteins were also immunoprecipitated with a mouse monoclonal anti-HA antibody and then western blotted with rabbit antiserum raised against a synthetic F_2 peptide. Panel A: lanes 1-5 were probed with mixture of anti-HeV G1 and anti-NiV G1 rabbit antisera; lanes 6-11 were probed with the anti-myc antibody. Panel B: lanes 1-5 were probed with the anti-F_2 antiserum; lanes 6-10 were first immunoprecipitated with the anti-HA antibody and then probed with the anti-F_2 antiserum. This experiment was done twice.


A.
kDA
WR
HeV G
HeV G myc
NiV G
NiV G myc
WR
HeV G
HeV G myc
NiV G
NiV G myc
80.9
63.8
32.4
26.0
B.
kDA
WR
HeV F
HeV F HA
NiV F
NiV F HA
WR
HeV F
HeV F HA
NiV F
NiV F HA
80.9
63.8
49.5
32.4
26.0
20.0

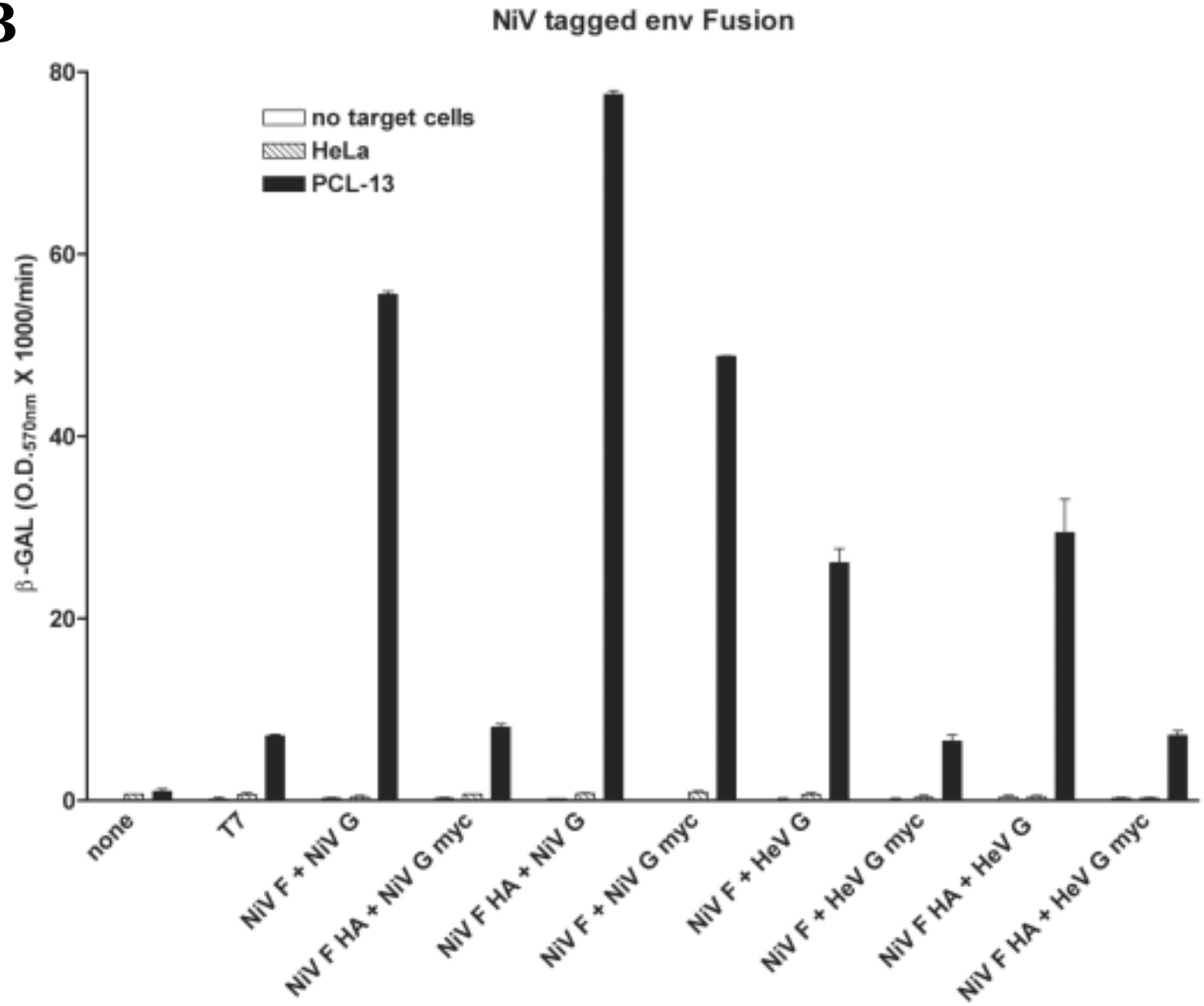
and epitope-tagged HeV and NiV F-HA was analyzed in a manner similar to that described for the G Western blots. However, the HA Mab did not detect the HA-tagged glycoproteins well by Western blot, a problem we have noted before in other tagged protein constructs. Therefore, lysates were immunoprecipitated with the HA Mab and the precipitates were separated by SDS- PAGE and then examined by Western blot analysis.. **Figure 25 Panel B** shows wild-type HeV and NiV F and epitope-tagged HeV and NiV F-HA. Lanes 1-5 were probed using the F2 peptide antiserum, lanes 6-10 were immunoprecipitated with the HA Mab and then probed with the F2 peptide antiserum. Both HeV F-HA and NiV F-HA migrated very similarly to the wild-type HeV and NiV F. Furthermore, the HA Mab specifically immunoprecipitated only the HA-tagged F constructs.

Functional activities of the epitope-tagged envelope glycoproteins of HeV and NiV. Prior to initiating in the co-immunoprecipitation studies it was important to determine the functional properties of these epitope-tagged envelope glycoproteins in comparison to their wild-type counterparts. We first analyzed the functional activities of our tagged constructs in various homotypic and heterotypic combinations, using singly tagged or dually tagged glycoproteins with the quantitative cell-fusion assay. We noted that the correlation of fusion activity was again consistent with the species of F employed. **Figure 26, Panel A** depicts the level of β-Gal activity produced in fusion reactions mediated by envelope combinations including HeV F or HeV F-HA with either species of G. The β-Gal activity observed in the presence of HeV F-HA was approximately one third of that seen in the presence of HeV F. Although HeV F-HA was functional, we

Figure 26. Quantitation of cell fusion mediated by HeV and NiV epitope-tagged envelopes. HeLa cells were infected with vaccinia recombinants encoding HeV F, HeV F-HA, HeV G, HeV G-myc, NiV F, NiV F-HA, NiV G and NiV G-myc glycoproteins in different combinations, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Each designated target cell type was infected with the *E.coli LacZ*-encoding reporter vaccinia virus vCB21R. The HeV or NiV epitope-tagged glycoprotein-expressing cells (1×10^5) were mixed with each target cell type (1×10^5) in duplicate wells of a 96-well plate. After 3 hr at 37°C, Nonidet P-40 was added and β-Gal activity was quantified. The level of background β-Gal activity in target cell populations alone is indicated as none, the level of background β-Gal activity in effector cell populations alone is indicated as no target cells. The β-Gal activity from target cells mixed with HeLa effector cells infected with only T7 RNA polymerase-encoding vaccinia virus and no vaccinia recombinants encoding NiV or HeV epitope-tagged glycoproteins is indicated as T7. A. HeV epitope-tagged envelope-mediated cell fusion. B. NiV epitope-tagged envelope-mediated cell fusion. This experiment was done 3 times.


A
HeV tagged env Fusion
no target cells
HeLa
U373
β-GAL (O.D.570nm X 1000/min)
400
300
200
100
0
none
T7
HeV F + HeV G
HeV F HA + HeV G myc
HeV F HA + HeV G
HeV F + HeV G myc
HeV F + NiV G
HeV F + NiV G myc
HeV F HA + NiV G
HeV F HA + NiV G myc

B

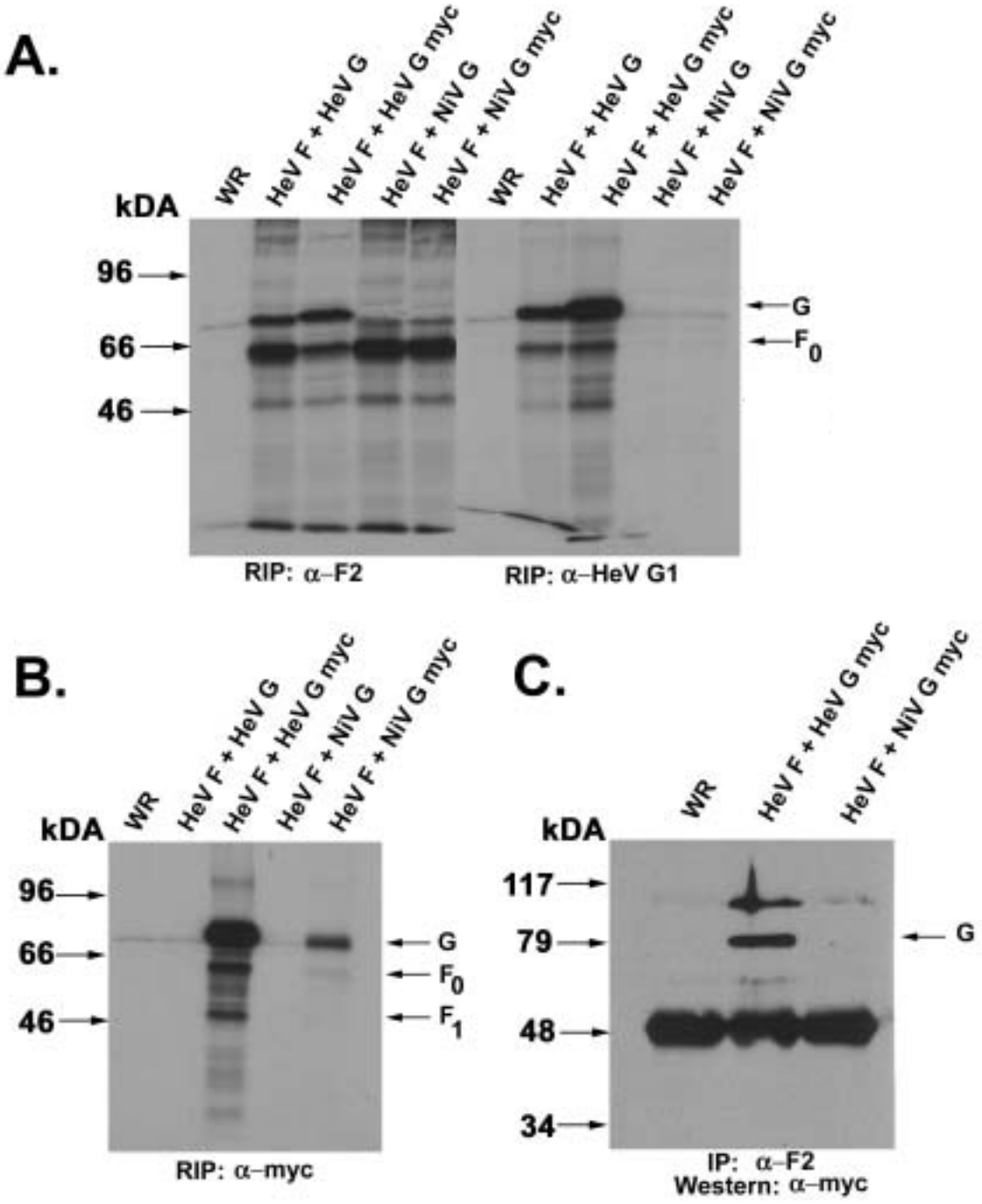

NiV tagged env Fusion
no target cells
HeLa
PCL-13
β-GAL (O.D.570nm X 1000/min)
80
60
40
20
0
none
T7
NiV F + NiV G
NiV F HA + NiV G myc
NiV F HA + NiV G
NiV F + NiV G myc
NiV F + HeV G
NiV F + HeV G myc
NiV F HA + HeV G
NiV F HA + HeV G myc

decided to exclude it from additional co-immunoprecipitation studies involving HeV F. Thus, due to its reduced fusion activity, additional envelope glycoprotein combinations involving HeV F-HA with either HeV G-myc or NiV G-myc were also not pursued. However, when HeV F was expressed in combination with HeV G-myc, their β-Gal activity was very similar to wild-type HeV G, and this was true for HeV F with either wild-type NiV G or NiV G-myc. In parallel, **Figure 26, Panel B** shows the levels of cell-fusion mediated by various envelope glycoprotein combinations that include NiV F or NiV F-HA with different species of G. An alternative target cell, PCL-13, a human head and neck carcinoma, was used because it was capable of mediating the highest fusion levels for both HeV and NiV, higher than previously reported with the human U373 cell line (data not shown). We speculate that this higher fusion activity is likely due to higher cell-surface expression of the virus receptor. In contrast to HeV F-HA, NiV F-HA was able to function quite well in mediating fusion. Indeed, NiV F-HA actually showed a degree of enhancement when compared to wild-type NiV F. The NiV G-myc protein was capable of functioning in a similar fashion to wild-type NiV G. When NiV F or NiV F-HA were used in heterotypic combinations with HeV G, both where capable of producing similar levels of cell-fusion. However, in combinations that contained a tagged version of both F and G, there were again variable reductions in fusion activities to as much as 50% depending on the combination. In contrast to HeV F-HA, the NiV F-HA construct retained unimpaired fusion activity when measured in the context of either wild-type HeV G or NiV G. The HeV G-myc and both NiV epitope-tagged envelope glycoproteins were functional in homotypic and heterotypic combinations when the other envelope glycoprotein was not tagged. The fusion potencies of each combination of an F and G

appeared to be dependent on the F glycoprotein employed. We next sought to analyze the interactions between homotypic and heterotypic combinations of F and G to determine whether the correlation between strong interaction between the envelope glycoproteins was consistent with more potent membrane fusion activity. We only used functional epitope-tagged envelopes for these studies.

Co-Immunoprecipitation of Hendra virus F with HeV or NiV G glycoproteins. Since NiV G-myc was capable of supporting membrane fusion with HeV F to the levels of wild-type HeV F and HeV G-myc combinations, we first examined the potential interaction between HeV F and NiV G-myc. Surprisingly, despite the retention of potent fusion with the HeV F in combination with either attachment protein, strong F and G interaction occurred only with HeV F and HeV G-myc. NiV G-myc was not efficiently co-immunoprecipitated with HeV F. **Figure 27** shows the co-immunoprecipitation results obtained from combinations of HeV F with different G species. In this experiment, the epitope-tagged envelope glycoproteins were produced in culture by infection of HeLa cells with the appropriate recombinant vaccinia virus. Beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine, and then chased for 2 hours with complete medium. Cell lysates were prepared as described earlier and subjected to immunoprecipitation with different antisera or Mabs. **Figure 27,** confirms the results shown previously in **Figure 23. Panel A** was precipitated with either anti–F2 antiserum, lanes 1-5, or anti-HeV G1 antiserum, lanes 6-10. HeV G-myc was capable of interacting with HeV F in a similar manner as observed with wild-type HeV G. It was also apparent that although HeV F is clearly precipitated in lanes 4 and 5,

Figure 27. Co-immunoprecipitation of HeV F with either HeV or NiV G or epitope-tagged HeV G-myc or NiV G-myc. HeLa cells were infected with HeV F with HeV or NiV G or HeV or NiV G-myc encoding viruses in different combinations and were incubated 16 h at 37°C. For radioimmunoprecipitation (RIP), beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine . For Western blot, infected cells were washed two hours post infection and incubated in medium overnight. Lysates were prepared in buffer containing 1%Triton X-100 and clarified by centrifugation. For RIP, lysates were incubated with rabbit anti-F_2 antiserum, rabbit α-HeV G1, or a commercial mouse α-myc antibody followed by Protein G-Sepharose. The metabolically labeled proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by fluorography. For Western blot, lysates were incubated with rabbit anti-F_2 antiserum followed by Protein G-Sepharose. The proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by chemiluminescence. Panel A: RIP: lanes 1-5 were immunoprecipitated with anti-F_2; lanes 6-10 were immunoprecipitated with rabbit anti-HeV G1. Panel B: RIP: lanes 1-5 were immunoprecipitated with anti-myc. Panel C:IP and Western: lanes 1-5 were immunoprecipitated with anti-F_2 and then probed using the mouse anti-myc antibody. This experiment was done 3 times.

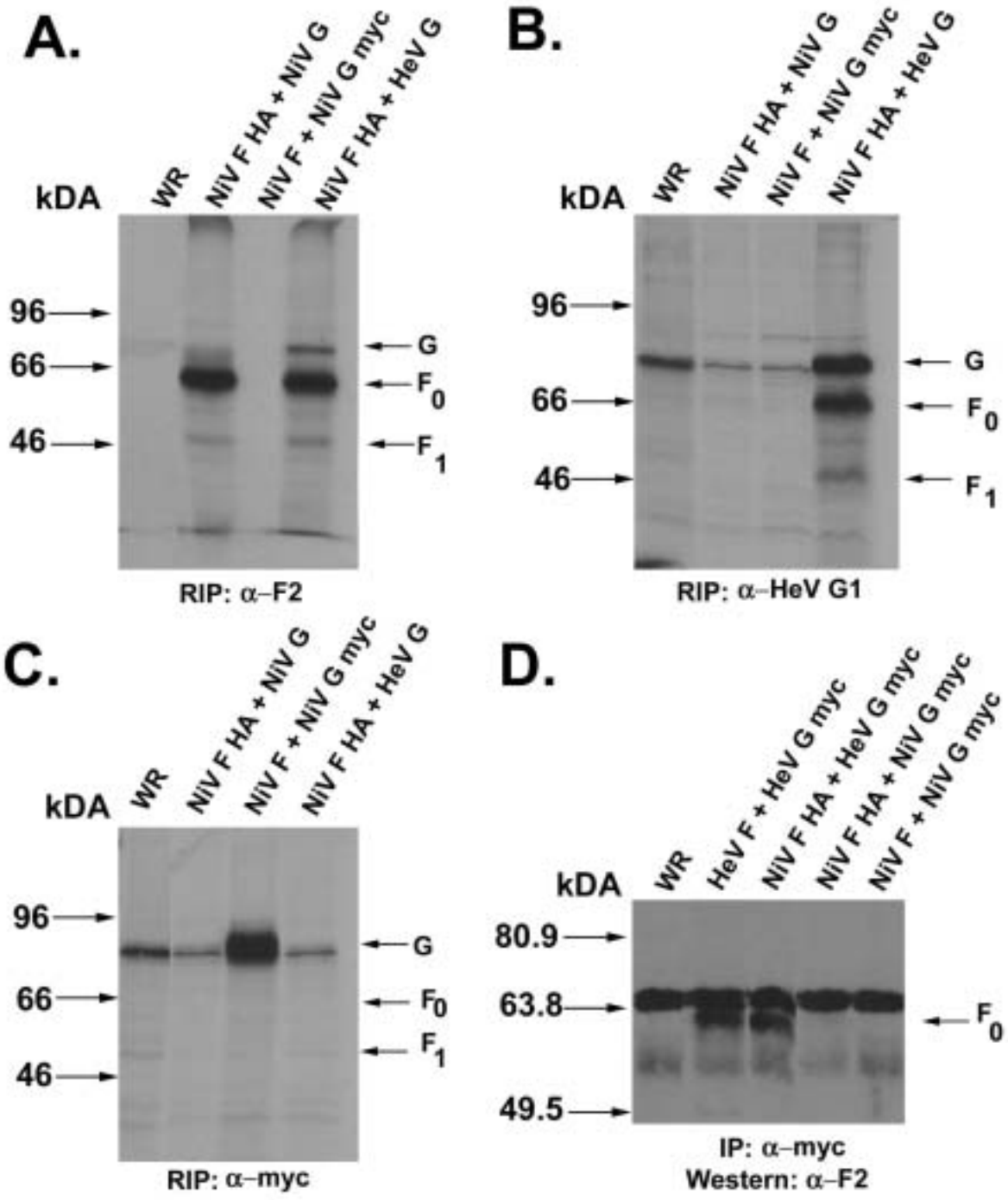

A.
WR
HeV F + HeV G
HeV F + HeV G myc
HeV F + NiV G
HeV F + NiV G myc
WR
HeV F + HeV G
HeV F + HeV G myc
HeV F + NiV G
HeV F + NiV G myc
kDA
96
66
46
G
F0
RIP: α–F2
RIP: α–HeV G1
B.
WR
HeV F + HeV G
HeV F + HeV G myc
HeV F + NiV G
HeV F + NiV G myc
kDA
96
66
46
G
F0
F1
RIP: α–myc
C.
WR
HeV F + HeV G myc
HeV F + NiV G myc
kDA
117
79
48
34
G
IP: α–F2
Western: α–myc

there is very little or no co-precipitation of NiV G or NiV G-myc. The very faint bands in lanes 4 and 5 that migrate just above F_0 may represent weak interactions between HeV F and NiV G or NiV G-myc. Since the anti-HeV G1 antiserum does not cross-react with NiV G, lanes 9-10 do not show any precipitated material and serve as an additional negative control. To examine more closely the interaction of HeV F with NiV G-myc, we immunoprecipitated additional lysate material with the anti-myc Mab. As Shown in **Figure 27, Panel B**, anti-myc Mab specifically precipitated HeV G-myc and NiV G-myc, shown in lanes 3 and 5. Again, the appearance of HeV F_0 and F_1 demonstrated the strong interaction between the HeV F and HeV G-myc glycoproteins. Conversely, F_0 is barely detectable in lane 5, again suggesting a very weak interaction between NiV G-myc and HeV F. As a final confirmation, the HeV F with different G complexes were immunoprecipitated and then analyzed by Western blot to confirm the presence of the HeV or NiV G-myc protein. As shown in **Figure 27, Panel C**, only HeV G-myc is detectable after immunoprecipitation with anti-F_2 antiserum. Taken together, these results do not support our original hypothesis that the strength of interaction between F and G correlates with the fusion activity seen mediated by HeV F. Although the HeV G-myc and NiV G-myc proteins mediate similar levels of membrane fusion with HeV F, HeV G-myc maintains a strong interaction with HeV F, while NiV G-myc has a weak interaction with HeV F under the experimental conditions used here. It is also important to point out that parallel co-immunoprecipitation experiments have been carried out using several non-ionic detergents and under less-stringent washing conditions, and these experiments yielded identical results. Further, these experiments included a washing step using a buffer containing 0.1% DOC / 0.1%SDS in addition to the 1% Triton X-100. The

inclusion of an ionic detergent wash increases the possibility that the interactions being measured are specific and not due to non-specific interactions or aggregations of proteins. Ionic wash experimental procedures for co-immunoprecipitation studies have been discussed and utilized by Morrison and colleagues in similar studies on the envelope glycoproteins of NDV (58).

Co-Immunoprecipitation of Nipah virus F with HeV or NiV G glycoproteins. In the companion analysis, where NiV F and NiV FHA were expressed in different combinations with G (**Figure 28**), we noted consistency with the results shown in **Figure 27**. Again, fusion activities were dependent on the F glycoprotein, and not the strength of its interaction with G. Homotypic NiV F-HA and G or NiV F and G myc do not strongly associate, whereas NiV F-HA and HeV G could be co-immunoprecipitated quite efficiently. **Figure 28** shows co-immunoprecipitation results with NiV F and NiV F-HA with different G species. The epitope-tagged envelopes were produced in culture by infection of HeLa cells with the appropriate recombinant vaccinia virus. Beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine, and then chased for 2 hours with complete medium. Cell lysates were prepared as before and immunoprecipitated with different antisera or Mab. Shown in **Figure 28, Panel A** shows lysates that were immunoprecipitated with anti-F2 antiserum. As expected, the anti-F2 antiserum was only capable of precipitating NiV F-HA containing complexes, and not NiV F containing combinations. Lane 4 clearly shows the appearance of HeV G, demonstrating that NiV F-HA is capable of interacting with HeV G. Again, we can detect a faint band in lane 2 which may represent the substantially weaker interaction

Figure 28. Co-immunoprecipitation of NiV F with either HeV or NiV G or epitope-tagged HeV G-myc or NiV G-myc. HeLa cells were infected with NiV F with HeV or NiV G or HeV or NiV G-myc encoding viruses in different combinations and were incubated 16 h at 37°C. For radioimmunoprecipitation (RIP), beginning at 6 h post-infection, the cells were labeled overnight with [^{35}S]-methionine/cysteine . For Western blot, infections were washed two hours post infection and incubated in medium overnight. Lysates were prepared in buffer containing 1%Triton X-100 and clarified by centrifugation. For RIP, lysates were incubated with rabbit anti-F_2 antiserum, rabbit anti-HeV G1, or a commercial mouse anti-myc antibody followed by Protein G-Sepharose. The metabolically labeled proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by fluorography. For Western blot, lysates were incubated with mouse anti-myc antibodies followed by Protein G-Sepharose. The proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by chemiluminescence. Panel A: RIP: lanes 1-5 were immunoprecipitated with anti-F_2; Panel B: lanes 1-5 were immunoprecipitated with rabbit anti-HeV G1. Panel C: RIP: lanes 1-5 were immunoprecipitated with anti-myc. Panel C:IP and Western: lanes 1-5 were immunoprecipitated with mouse anti-myc antibody and then probed using anti-F_2 rabbit antiserum. HeV F + HeV G-myc was included as a positive control. This experiment was done 3 times.


A.
WR
NiV F HA + NiV G
NiV F + NiV G myc
NiV F HA + HeV G
kDA
96
66
46
G
F0
F1
RIP: α–F2
B.
WR
NiV F HA + NiV G
NiV F + NiV G myc
NiV F HA + HeV G
kDA
96
66
46
G
F0
F1
RIP: α–HeV G1
C.
WR
NiV F HA + NiV G
NiV F + NiV G myc
NiV F HA + HeV G
kDA
96
66
46
G
F0
F1
RIP: α–myc
D.
WR
HeV F + HeV G myc
NiV F HA + HeV G myc
NiV F HA + NiV G myc
NiV F + NiV G myc
kDA
80.9
63.8
49.5
F0
IP: α–myc
Western: α–F2

between NiV F-HA and NiV G. The materials shown in **Figure 28, Panel B** were immunoprecipitated with anti-HeV G1, and the clear appearance of F_0 and F_1 demonstrates that HeV G can associate with NiV F-HA. Since the NiV G-myc protein is functional in the fusion assay, we also examined the NiV F + NiV G-myc combination immunoprecipitated with anti-myc Mab as shown in **Figure 28 Panel C**. There was no detectable NiV F in lane 3, which further indicated that NiV F could not interact with NiV G-myc. To ensure specificity, Western blot analysis was performed, shown in **Figure 28, Panel D**. The HeV F and HeV G-myc were included as positive controls. Lysates were first immunoprecipitated with anti-myc Mab, and following precipitation and processing, the material was separated by SDS-PAGE and analyzed by Western blot with the anti-F_2 antiserum. Lane 5 of **Panel D** further demonstrates that there is no detectable interaction between NiV F and NiV G-myc. Finally, lanes 3 and 4 show the results obtained with the double-tagged envelope combinations, which were previously shown to be variably impaired in their abilities to mediate cell-fusion. The NiV F-HA and HeV G-myc glycoprotein combination (lane 3) demonstrates a detectable interaction, whereas the NiV F-HA and NiV G-myc glycoprotein combination shows no detectable interaction (lane 4).

In summary, the NiV F-HA protein can interact with HeV G protein, but it does not interact with the NiV G protein. The detection of a physical interaction between the NiV F or F HA glycoprotein with different G species did not correlate with the membrane fusion potential of the particular envelope combination. NiV F or NiV F-HA were capable of fusing to the same extent with NiV G-myc or HeV G-myc, and this fusion activity was unrelated to a detectable interaction between the envelope

glycoproteins. Therefore, our findings indicate that there does not appear to be a correlation between the strength of the physical interaction between the fusion and attachment envelope glycoproteins and their membrane fusion activity. These observations are in contrast with certain observations by Plemper et al. Their analysis of MeV F and H envelope glycoproteins revealed that disruption of the strong interaction between F and H by insertion of a cytoplasmic epitope tag onto H, was correlated to an increase neutralization sensitivity by soluble CD46 (a MeV receptor) as well as an increase in their fusogenicity (138).

Their are two central models proposed for the role of the attachment glycoprotein in the paramyxovirus-mediated membrane fusion process (**Figure 29**), recently reviewed and detailed by Morrison and colleagues (139). Our observations of the biochemical and functional properties of the HeV and NiV envelope glycoproteins are consistent with the notion that F and G are pre-associated (Model 2). In this model, receptor engagement induces conformational alteration in the attachment glycoprotein, and presumably, this process alters or releases an interaction with F. F can then proceed towards its fusion active state; formation of the 6-helix bundle and membrane merger. Although a strong interaction between NiV F and G was not observed, we favor model 2 and speculate that the interaction between NiV F and G at the resting (non-receptor engaged) state is too weak to survive the co-immunoprecipitation assay. Since neither HeV nor NiV F can mediate membrane fusion alone, it is likely that the G protein has to trigger specific conformational changes in F, not just simply release it.

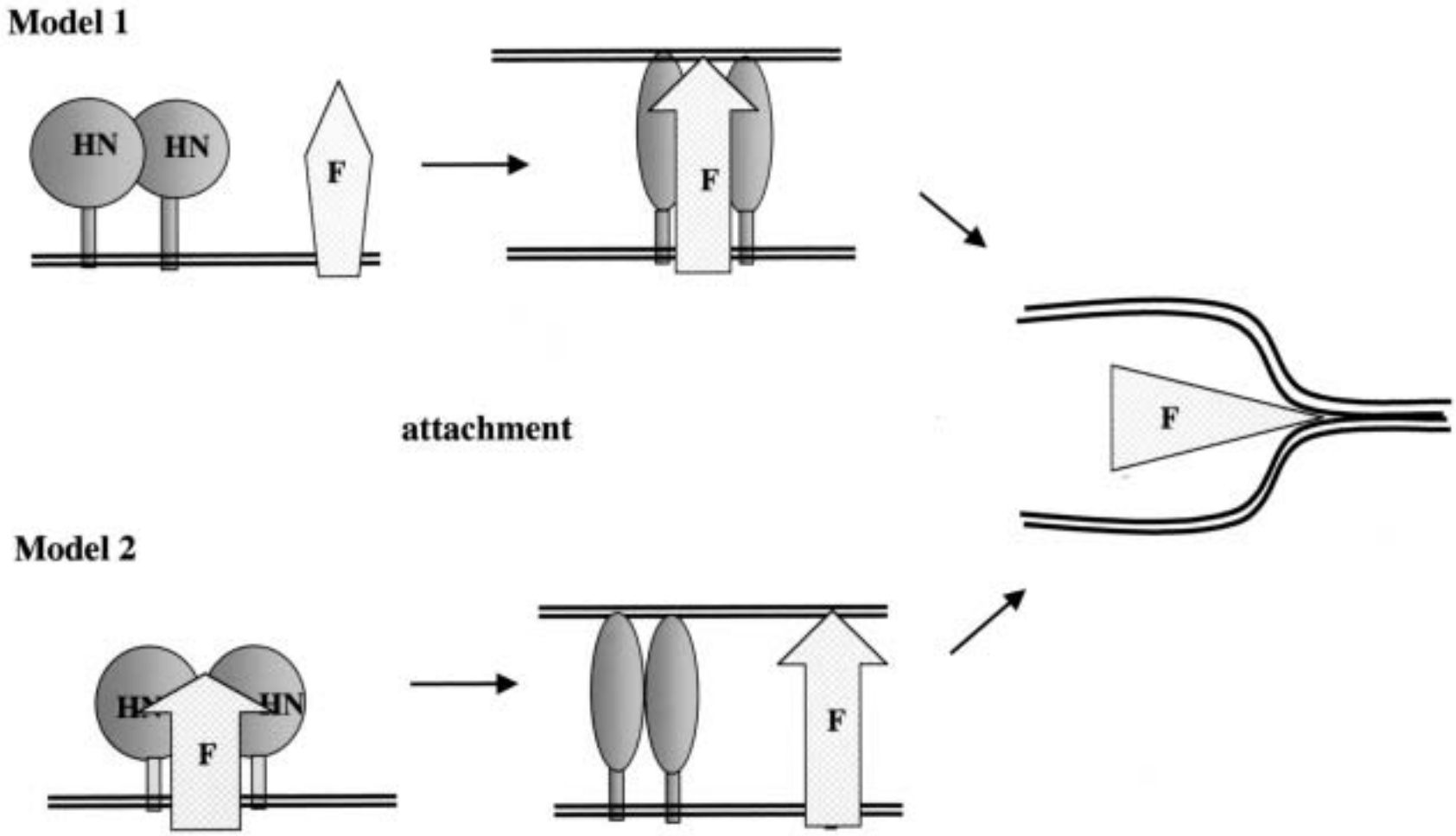


Figure 29. Models of Paramyxovirus membrane fusion ((139) Figure 9, with permission).

DISCUSSION

The results here have established the requirements for henipavirus co-immunoprecipitation of the envelope glycoproteins, and have defined differences between the HeV and NiV attachment envelope glycoprotein. To our surprise, we also demonstrated that the strength of interaction between the F and G did not correlate with fusion activity. Nevertheless, these observations and properties of the HeV and NiV envelope glycoproteins may be extremely advantageous to dissecting the domain of G which mediates its association with F. Because either HeV or NiV F interacts strongly with HeV G, it could be surmised that the domain or elements in F that mediate interaction with G are in fact conserved between the two viruses. From studies to date, this domain may encompass a central region of F between HR1 and HR2 that upon close examination reveals a heptad repeat like structure (140, 141) and has been referred to as HR3. The HeV and NiV F are highly conserved in this region one V292I conservative substitution. In regards to the attachment glycoproteins of paramyxoviruses, studies to date on NDV, SeV and PIV have indicated that regions in both the globular head and stalk regions of the HN glycoprotein are important in promoting fusion. The majority of evidence points to the stalk region (58, 142-150). The stem region of the attachment glycoprotein encompasses a small heptad domain element spanning residues ~74-110, which was first noted in the HN of NDV upon close inspection of this region by Stone-Hulslander and Morrison (148). This element consisted of a series of 6 hydrophobic residues in the a position of a helical wheel which are spaced by a 7 amino acid stretch. Analysis of this region by secondary structure-prediction software (148) indicated a potential to form an α-helical structure. Several mutations within this putative heptad

domain element (amino acids 74-110) of HN of NDV, which did not alter the expression profile of HN nor its ability to be recognized by conformation-dependent MAbs, were noted to impair its ability to support F-HH mediated membrane fusion significantly. These observations suggested that the important element in the attachment glycoprotein, at least of NDV HN, is this heptad domain structure. Perhaps, it mediates F interaction by association with one of the heptad domains in F (148). Most recently, it has been noted that the NDV HN glycoprotein can clearly influence the conformation of F, as measured by the accessibility of the HR1 F domain to specific antibody binding (139). Here the coexpression of HN with F allows antibody to interact with HR1 of F. In contrast, this domain is not accessible to antibody when F is expressed in the absence of HN. These observations support the paramyxovirus fusion model, which suggests the fusion and attachment glycoproteins in the resting state (not engaged with cellular receptors) are physically associated. Stone-Hulslander and Morrison speculate that the attachment glycoprotein is holding F in its non-fusogenic conformation, and only upon receptor engagement and conformational change in the attachment glycoprotein is F then released to undergo conformational changes leading to 6-helix bundle formation and facilitation of membrane fusion. This would be in accord with the observation that paramyxovirus F expressed alone neither mediates fusion (with the exception of SV5 under certain conditions) nor has an inaccessible HR1 domain. This is perhaps because it has transitioned to a partially fusion-triggered conformation at an inappropriate moment.

Future studies aimed to identify the region of HeV G that interacts with F have already begun in the laboratory, and these studies are focused on designing a panel of chimeric G proteins between HeV and NiV. The HeV and NiV G envelope glycoproteins

are 78.4 % similar. The globular head domains of HeV and NiV G (~amino acid 155 to 605) are 77.7 % similar, and the extracellular stem regions (~amino acid 74-155) are 85.4 % similar. Initial strategies include the design of 6 chimeras, one chimeric pair will have the globular head domain swapped between the two proteins. A second chimeric pair will exchange the extracellular stem regions between each of the G glycoproteins. The final pair will have only part of the globular head exchanged between the two proteins. Based on the available data in the literature, we hypothesize that swapping the HeV stem domain into the NiV G backbone will generate a chimera, which will posses an enhanced ability to interact and co-immunoprecipitate either NiV or HeV F. If we are correct in identifying this stem region of G as the important domain that facilitates F interaction, it will further support previous data already mentioned above, and the proposed model of paramyxovirus fusion and attachment envelope glycoprotein interaction. Moreover, these data will be from more divergent viruses, which have attachment glycoproteins that lack hemagglutinating and neuraminidase activity and do not employ sialic acid receptors for virus binding and entry. If we are incorrect and another or unexpected domain is identified which facilitates F interaction, it will be a completely new observation that will add to the distinct differences of these viruses as compared with other members of the paramyxovirus family.

Once we have located the domain of interaction, we would focus on point-mutagenesis strategies in the smallest domain(s) elements that are indicated as important for F interaction. For example, if the stem region is defined as the important element, and it significantly overlaps with a potential heptad-like domain as noted in NDV HN, we would disrupt this putative helical heptad element by substitution mutation of the

hydrophobic residues in the potential ‘a’ positions. This detailed analysis will help shed light on the complicated mechanism of how the attachment glycoprotein plays a key role in promoting membrane fusion mediated by the F protein.

Because NiV F-mediated fusion was not rescued to levels of HeV F-mediated fusion when employed in a heterologous combination with HeV G, we presume there is another functional domain that differs from the G interaction domain, and the HR1 and HR2 domains. This novel functional domain is hypothesized to affect fusion activity and thus account for the different fusion activities of the HeV and NiV F glycoprotein. To pursue these studies, chimeric F proteins will have to be constructed and tested using the quantitative fusion assay.

The work presented here has opened a new avenue to explore the details of how the interaction between the attachment and fusion proteins promotes the membrane fusion of paramyxoviruses. We have developed a system for detecting the co-immunoprecitiates, and equally important, we have HeV and NiV G, which are only 21.6 % divergent, as a place to start “domain-swapping” studies. Once these domains have been mapped thoroughly, they will offer new insights into paramyxovirus fusion mechanisms, which in turn will give rise to many new questions. Perhaps these domains give rise to the type-specific restrictions of the fusion and attachment envelope glycoproteins within the *Paramyxoviridae*. Equally important will be to discern how these regions play a role in promoting membrane fusion.

Chapter 6

Discussion

Emerging paramyxoviruses.

Hendra and Nipah virus are just two of many novel emerging paramyxoviruses. In the last twenty years, more than 10 new paramyxoviruses have been identified from all different regions of the world, and these are summarized in Table 4. Phocine distemper virus, a closely related morbillivirus, has caused numerous epidemics in seal populations since its discovery in 1988. Experimental evidence suggests that environmental pollution may have been the biggest risk factor that contributed to the emergence of and the severity and extent of these infections in recent years. Menangle and Tioman viruses were discovered in Australia in 1997 and Malaysia in 1999, respectively, and both are viruses of fruit bats (flying foxes) in the genus Pteropus, (151, 152). Menangle virus caused of outbreak of reproductive disease in pigs. Tioman virus was discovered during the search for the natural reservoir of Nipah virus, and to date, it has not been shown to cause disease. Although classified as Rubulaviruses, these viruses were discovered on the same continents and in the same time frame as Hendra and Nipah viruses, respectively. Similar to Hendra and Nipah viruses, the attachment glycoproteins of these new rubulaviruses lack important determinants of neuraminidase activity which suggests that they are unlikely to possess the same degree of neuraminidase activity characteristic of other rubulavirus and respirovirus HN proteins. The mode of transmission of Menangle virus among pigs remains unknown (153). The flying fox has a very large range, and possesses the capability to carry these novel paramyxoviruses as far west as the Eastern coast of Africa. This presents the possibility that these viruses, and closely

Table 3. Emerging Paramyxoviruses

Virus	Genus	Year	Location	Isolation
Tupaia paramyxovirus	Unassigned	1979	Thailand	Tree shrew
Mapuera virus	Rubulavirus	1979	Brazil	Bat
Porcine rubulavirus	Rubulavirus	1984	Mexico	Pig
Phocine distemper virus	Morbillivirus	1988	Europe	Seal
Cetacean morbillivirus	Morbillivirus	1990	Europe	*
Salem virus	Unassigned	1992	USA	Horse
Hendra virus	Henipavirus	1994	Australia	**
Menangle virus	Rubulavirus	1997	Australia	Pig/bat(?)
Nipah virus	Henipavirus	1999	Malaysia	***
Tioman virus	Rubulavirus	1999	Malaysia	Bat
Un-named	Unassigned	2001	USA	Pig

* Porpoise/dolphin

** Horse/human/bat

*** Pig/human/bat

related viruses, share the same reservoir such as Hendra and Nipah and will emerge in new parts of the world.

Hendra and Nipah Virus Tropism

Most paramyxoviruses are highly species-specific and do not infect multiple species. Hendra and Nipah virus are unique in their ability to cause severe and fatal disease in several animal species and humans. The underlying reasons for how these viruses are able to mediate cross-species transmission remains unknown. HeV and NiV are classified as zoonotic, biosafety level 4 (BSL-4) agents and, thus, their manipulation under laboratory conditions is highly restricted. By adapting our cell-cell fusion reporter-gene assay to the HeV- and NiV-mediated fusion system we were able to examine systematically and a quantitatively, a battery of target cell populations representing a variety of cell types and animal species. HeV and NiV envelope glycoproteins were capable of mediating fusion with many of the cell types and animal species tested. In fact, only a handful of receptor negative cells have been identified. Moreover, all of our studies thus far demonstrate that HeV and NiV were found to use the same receptor recognition pattern amongst the cell lines we examined, and those that supported the highest level of HeV-mediated fusion also supported the highest level of NiV-mediated fusion. These data support the hypothesis that HeV and NiV are likely using the same receptor for virus entry into receptive host cells. Our initial characterization of the HeV receptor demonstrated that it was sensitive to cleavage by trypsin, suggesting it was most likely a cellular protein. These two similar viruses may both mediate cross species

transmission due to expression of this cellular protein receptor in pigs, horses and humans.

Receptor Identification

Receptor identification was not an original specific aim of this thesis. However, we have significant evidence that supports the discussion and implementation of a receptor identification strategy that uses specific cDNA expression for the rescue of HeV and NiV-mediated membrane fusion. Our data demonstrates specific fusion-negative cell lines (in some cases from the same species), and protease sensitivity (destruction) of fusion-permissive cell line. Our collaborator's findings from a virus overlay protein-binding assay (VOPBA) and radio-immunoprecipitation assay have indicated that HeV and NiV bind predominantly to membrane proteins in the 35-50 kDa range. These observations strongly indicate a surface-expressed protein is serving as the virus receptor. In addition, treatment of membrane proteins with neuraminidase has had no effect on virus binding. The latter observation is consistent with the fact that removal of sialic acid from Vero cells (the cell line used to propagate live virus stocks) by neuraminidase treatment did not abrogate infection by HeV. Taken together, these findings strongly suggest that the cellular receptor for these viruses is a surface-expressed protein(s).

We have employed a novel cDNA screening technique using vaccinia virus-based cDNA libraries coupled with a newly developed modified vaccinia virus Ankara (MVA)-based reporter-gene assay system. The MVA-based reporter-gene assay system and recombinant MVAs expressing the HeV envelope glycoproteins are in the process of being constructed in the laboratory. The vaccinia-based cDNA libraries were provided

by Vaccinex, Inc. One of these libraries was derived from the PCL-13 cell line, the cell line with the highest HeV- and NiV-mediated fusion. By using non-replicating MVA for expression of T7 polymerase, T7-lacZ, and the envelope glycoproteins in our cell-cell fusion assay, the recombinant vaccinia viruses from the cDNA library will be the only replicating virus in reporter-gene assay system and offers a means of selecting and amplifying a fusion-permissive cDNA recombinant from the library. Using one or more NiV and HeV receptor-negative cell lines, the system will identify the cellular receptor by restoration of membrane fusion through the expression of a specific cDNA. Membrane fusion events will be identified using overlays that contain X-Gal that in the presence of β-Gal will turn blue. We have already constructed MVA versions of HeV F and G, and have obtained two versions of an MVA-T7 RNA polymerase-encoding virus from G. Sutter (T7-linked to *p*7.5) (154) and G. Kovacs (T7-linked to the strong synthetic E/L, unpublished). We are presently working on construction of an MVA-based T7- β-Gal reporter virus.

If we are successful in identifying a functional receptor molecule, one that confers membrane fusion permissiveness to a negative cell line, there will be a logical progression of experiments to validate such a finding and answer questions that relate to viral tropism. Firstly, is the receptor known? Is it a member of a larger gene family, and is this related to the observed broad host range of these viruses? Are there any available antibodies which could be tested for their ability to block cell-fusion. If so, do such antibodies block live virus infection (experiments to be performed by our collaborators). Second, does the receptor-encoding vaccinia virus confer fusion permissiveness to all HeV-fusion negative cell lines? Is NiV-mediated fusion also rescued? Can a receptor

transduced cell line confer live virus replication? The identification of the HeV and NiV receptor would have many important implications and is a very exciting area of our ongoing research. Knowing the nature of the cellular receptor(s) could make them targets for antiviral agents, and enable an enhanced understanding of *in vivo* viral pathogenesis.

Antiviral Drugs

There are currently no vaccines or therapies for treating HeV- or NiV-infected individuals. In 1999, the only way to ensure containment and prevent further spread of Nipah virus was to cull over one million pigs. With a mortality rate near 40%, it is of the utmost importance to develop antiviral therapies against these agents. Considerable advances in the understanding of the structural features of these oligomeric viral envelope glycoproteins has been attained in recent years and have centered on the influenza virus and HIV systems. A notable structural feature of many of these fusion glycoproteins is the presence of 2 α-helical domains referred to as "heptad repeats". These repeats are important for both oligomerization and function of the glycoprotein, and they appear to be involved in the formation of a "trimer-of-hairpins" structure (63). Peptides corresponding to either of these domains can potently inhibit the fusion process as was first noted with sequences derived from the gp41 subunit of HIV-1 envelope glycoprotein (65, 66). Indeed, the development and clinical application of fusion-inhibitors as antiviral therapies for HIV-1 has been a direct result from this area of research. In the present study we analyzed the heptad repeats of HeV and NiV F using helical wheel diagrams. We identified the sequences that would be likely inhibitors of HeV and NiV-mediated fusion based on sequence similarity to the SV5 F glycoprotein and known C-terminal heptad-derived

peptide inhibitors of SV5 (68). The HeV FC2 and NiV FC1 peptides completely inhibited either HeV- or NiV-mediated fusion in the nM range. These peptides are presently being evaluated as inhibitors of live HeV infection under BSL-4 conditions, and may represent a therapeutic avenue for both HeV and NiV infections. Preliminary data using HeV FC1, our first synthesized peptide analogous to the C-terminal heptad of HeV F, are shown in Table 4.

Paramyxovirus fusion

Unexpectedly, we demonstrated that the strength of interaction between the F and G envelope glycoproteins of HeV and NiV did not correlate with fusion activity. Nevertheless, these observations and properties of the HeV and NiV envelope glycoproteins may be extremely advantageous to dissecting the domain of G that mediates its association with F, an event thought to be key in the model of paramyxovirus fusion. We have already begun construction of chimeric G proteins to map the domain that interacts with the F envelope glycoproteins. Once mapped, domains can be swapped and the ability of NiV G to interact with HeV or NiV F may be restored. Moreover, this type of analysis may be expanded to include other members of the *Paramyxoviridae* and might lead to the mapping of specific regions of the fusion and attachment protein that give rise to type-specific functional envelope glycoprotein combinations. A more refined mutagenesis strategy of the domains involved in mediating fusion and attachment glycoprotein interactions should shed light on the mechanism whereby the attachment protein is able to promote fusion.

Table 4. IC_{50} of HeV FC1 against live HeV infection

Peptide concentration	# Virus-infected foci	# Nuclei per Focus / syncytium	Min % decrease in # nuclei per syncytium
112 uM	27	1.2	97.3
56 uM	33	1.26	97.15
14 uM	35	7.06	84.0
3.5 uM	39	8.96	79.4
875 nM	39	15	66.08
219 nM	43	16.33	63.1
55 nM	**41**	**20.39**	**53.89**
No peptide	41	>44.22	

Virus was adsorbed to cells 30 mins before addition of peptide. Peptide was present during subsequent 24 hr incubation. These data are unpublished and shown here with permission from our collaborators.

Of the two central models proposed for the role of the attachment glycoprotein in the paramyxovirus-mediated membrane fusion process, (reviewed by Morrison and colleagues (139)), our observations are consistent with the notion that F and G are pre-associated (model 2). In this model, receptor engagement induces conformational alteration in the attachment glycoprotein, which in turn allows F to proceed towards its fusion active state; i.e., formation of the 6-helix bundle and facilitating membrane merger. We favor model 2 and speculate that the interaction between NiV F and G at the resting (non-receptor engaged) state is too weak to survive the co-immunoprecipitation assay. Ultimately, if we identify the HeV and NiV cellular receptor (s), we may be able to trigger a stronger detectable interaction between the NiV F and G envelope glycoproteins. We also may be able to mimic such a trigger by expressing both the NiV F and G envelope glycoproteins in receptor-positive cells and then determine if NiV F and NiV G can associate to a detectable degree in the presence of receptor. The same type of experiment could be done with HeV F and NiV G as well. This may provide more evidence that HeV and NiV G share the same receptor.

Finally, the work presented here does not explain the enhanced fusion activity of the HeV F envelope glycoprotein as compared to NiV F. In preliminary studies, we mutated HeV F at position 109 to give rise to a K109R mutation. This mutation altered the basic amino acid just prior to the putative cleavage site of F_2 from Lys to Arg as seen in NiV F. This mutation did not affect the fusion activity of HeV F (data not shown) and did not explain the enhanced fusion of HeV F as compared to NiV F. We also speculated that an enhanced interaction between the F and G envelope glycoproteins may have explained the HeV F enhanced fusion activity, but we now know that this is not true

either. Future studies are aimed at designing chimeric HeV and NiV F glycoproteins in order to map the domain of HeV F that is responsible for the enhanced fusion activity. This type of analysis should help dissect any differences that arise after the interaction of F with attachment protein and before the formation of the six-helix bundle leading to membrane fusion.

Chapter 7

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